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## TOPICAL REVIEW

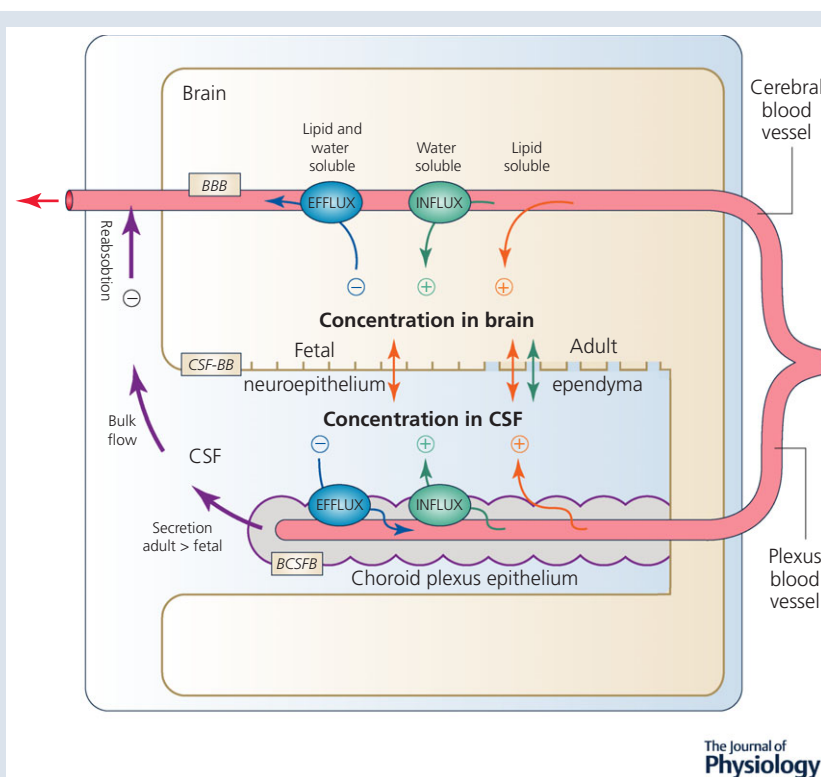
# Physiology and molecular biology of barrier mechanisms in the fetal and neonatal brain

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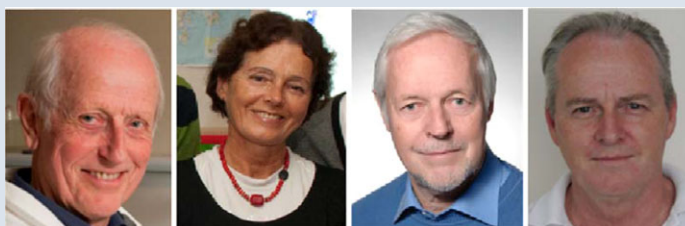
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**Abstract** Properties of the local internal environment of the adult brain are tightly controlled providing a stable milieu essential for its normal function. The mechanisms involved in this complex control are structural, molecular and physiological (influx and efflux transporters) frequently referred to as the 'blood–brain barrier'. These mechanisms include regulation of ion levels in brain interstitial fluid essential for normal neuronal function, supply of nutrients, removal of metabolic products, and prevention of entry or elimination of toxic agents. A key feature is cerebrospinal fluid secretion and turnover. This is much less during development, allowing greater accumulation of permeating molecules. The overall effect of these mechanisms is to tightly control the exchange of molecules into and out of the brain. This review presents experimental evidence currently available on the status of these mechanisms in developing brain. It has been frequently stated for over nearly a century that the blood–brain barrier is not present or at least is functionally deficient in the embryo, fetus and newborn. We suggest the alternative hypothesis that the barrier mechanisms in developing brain are likely to be appropriately matched to each stage of its development. The contributions of different barrier mechanisms, such as changes in constituents of cerebrospinal fluid in relation to specific features of brain development, for example neurogenesis, are only beginning to be studied. The evidence on this previously neglected aspect of brain barrier function is outlined. We also suggest future directions this field could follow with special emphasis on potential applications in a clinical setting.

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**Abstract figure legend** Barrier mechanisms in the developing brain. Blood–brain barrier (BBB), Blood–CSF barrier (BCSFB) and CSF–brain barrier (CSFB).

**Gene abbreviations: (For gene products (proteins), capitals are used.)** *Cacn*, calcium voltage-gated channel; *Clnka*, chloride voltage-gated channel Ka; *Clic*, chloride channel; *Gypa*, glycoporphin A; *Kcnh8*, potassium voltage-gated channel subfamily H member 8; *Kcnk9*, potassium two pore domain channel subfamily K member 9; *Cacn*, calcium voltage-gated channel; *Kcnmb1*, potassium calcium-activated channel subfamily M regulatory beta subunit 1; *Kcnmb2*, potassium calcium-activated channel subfamily M regulatory beta subunit 2; *Scn*, voltage gated sodium channels; *Scn1g*, sodium channel, nonvoltage-gated 1 gamma; *Sparc*, Secreted protein acidic and rich in cysteine; *vamp*, vesicle-associated membrane proteins; *Tmc5*, transmembrane channel-like 5; *Trpv*, transient receptor potential cation channel; *Wnt*, wingless-type MMTV (mouse mammary tumor virus) integration site.

## Introduction

The internal environment of the adult brain is defined and controlled by a series of mechanisms usually referred to colloquially as the blood–brain barrier. However, these are not unified identities, either in terms of their anatomical situation and structure, or in terms of their functional properties. The multifactorial characteristics of brain–blood interfaces are the main reason why misconceptions and controversies continue in the field (see box). One of the main areas of dispute has been the so-called 'immaturity' of brain barriers, especially the blood–brain barrier proper (cerebral endothelial interface), in the developing brain.

It has been widely believed for nearly a century that these mechanisms in the fetal and newborn brain are absent or poorly developed. This belief appears to stem

from misunderstandings and mistranslations of early studies, not helped by some poorly designed experiments (reviewed in detail in Saunders *et al.* 2014). In spite of extensive evidence to the contrary, this belief still persists (Allen, 2015; Oberdick *et al.* 2016; Panfoli *et al.* 2016; Amaraneni *et al.* 2017) with some new inventive terms appearing that imply some level of dysfunction: 'inefficient' (Panfoli *et al.* 2016), 'primitive' (Zhao *et al.* 2015). In place of this rather un-illuminating view of blood–brain barrier mechanisms in the developing brain, we propose that the specific barrier mechanisms present at any particular stage of brain development are ones that are appropriate for that stage of its development. They can thus be investigated in relation to specific features of brain maturation, such as neurogenesis (Lehtinen & Walsh, 2011; Lehtinen *et al.* 2013; Lun *et al.* 2015).

### Misconceptions and controversies discussed below

- Continued use of the term 'immature' to indicate a functional deficiency in brain barrier mechanisms.
- Do the first blood vessels that grow into the brain anlage have functionally effective intercellular tight junctions?
- Does increased apparent permeability (leakiness) to dyes and small molecular mass markers reflect 'breakdown' of tight junctions?
- Does greater accumulation of a molecule in developing brain and cerebrospinal fluid reflect greater permeability of barriers in the developing brain?
- What is the role of astrocytes in formation (initiation) and maintenance of blood–brain barrier properties?
- Permeability of the paracellular pathway.

In support of this proposition we summarize data that have accumulated over the past few decades from studies using classical physiological approaches and in more recent years from the application of molecular techniques. We also outline some key points in the biology of the field that are still controversial (box above) and indicate where this vital research may take us in the future.

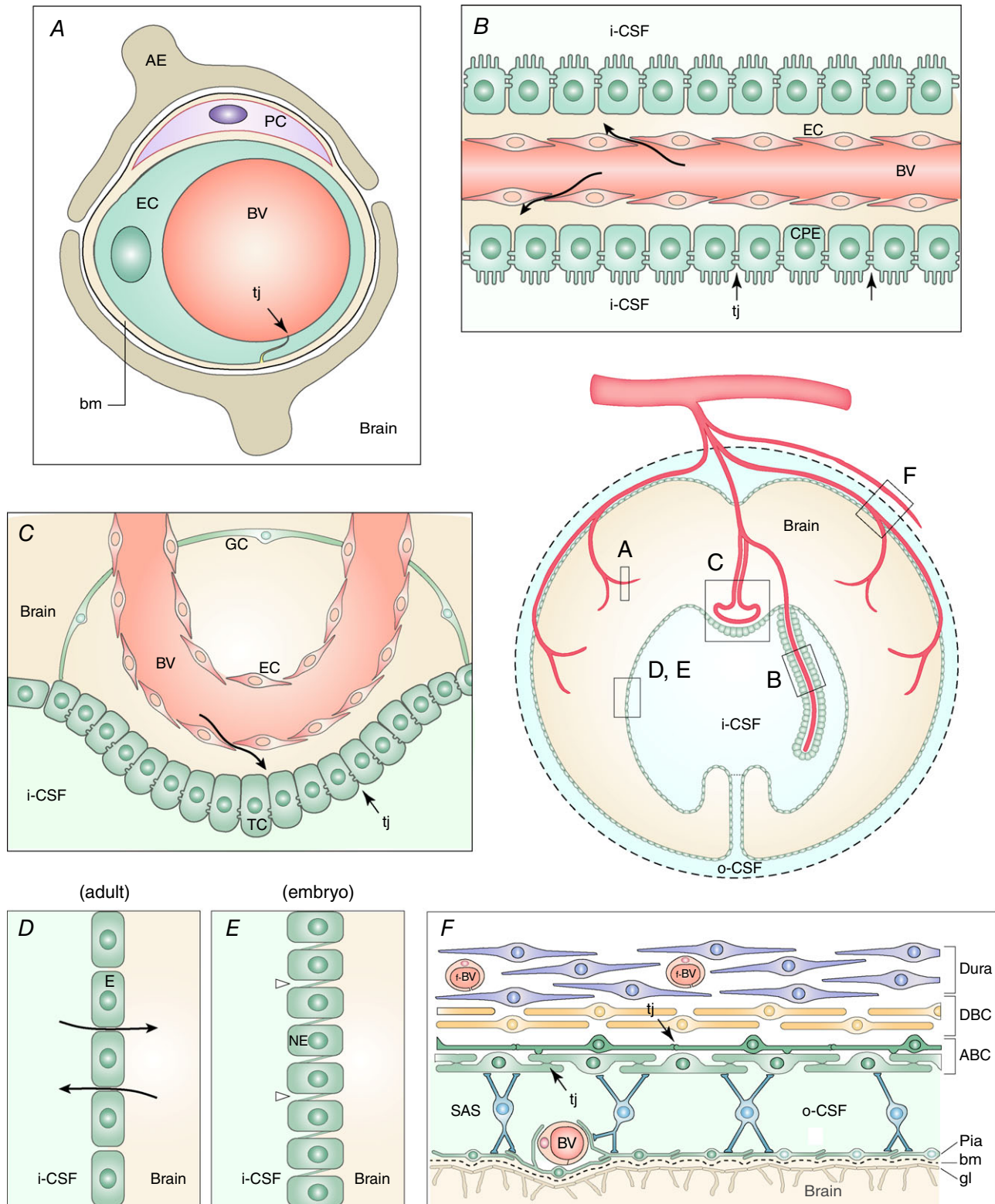
### Barrier interfaces between the brain and the rest of the organism

The fundamental structural component of almost all of the barrier interfaces is the tight junctions between the cells forming the interface. There are at least six identifiable barrier interfaces in the brain (including one that is exclusive to the fetal brain). These are illustrated and described in Fig. 1, with morphological details in Fig. 2. In summary these barrier interfaces are:

- (a) The blood–brain barrier (Figs 1A and 2Aa and b) is situated between the lumen of cerebral blood vessels and brain parenchyma. Tight junctions are present between the endothelial cells restricting permeability of the paracellular cleft (Brightman & Reese, 1969) to an extent that is still controversial (see below); however, the tight junctions in blood vessels of early developing brain appear to be impermeable to even very small molecules (Fig. 2A). Additional details are in legends to Figs 1 and 2.
- (b) The blood–cerebrospinal fluid barrier (Fig. 1B) in the choroid plexus within each brain ventricle. The barrier forming cells are the epithelial cells, which have tight junctions at the apices of adjacent epithelial cells forming the structural basis of this barrier; they prevent penetration of even small molecules from blood to cerebrospinal fluid (CSF; Fig. 2B). The blood vessels in the stroma of the choroid plexus are fenestrated and are thought not to form a barrier, although their endothelial cells appear to contain some key efflux

transporters that prevent entry of many lipid-soluble molecules into the brain and CSF (Møllgård *et al.* 2017). Extracellular matrix basement membrane may also contribute, but there are no astrocytic endfeet or pericytes.

- (c) Circumventricular organs (Fig. 1C). These comprise the organum vasculosum laminae terminalis (OVLT), subfornical organ, median eminence, subcommissural organ (SCO)–pineal complex and area postrema. Apart from the SCO, which has a normal blood–brain barrier, blood vessels in the circumventricular organs have similar permeability characteristics to vessels elsewhere in the body. This allows feedback penetration of circulating peptide hormones. However, these and other molecules are prevented from entering the CSF by tanycytes, connected by tight junctions between their apices; the perivascular space is separated off by tight junctions from the CSF milieu of the adjacent neuropil, as illustrated in Fig. 2Ca and b for the subcommissural organ (see Madsen & Møllgård, 1979).
- (d) Ependyma in adult brain (Figs 1D and 2D). Ependymal cells are linked by gap junctions; there is unrestricted exchange of even large molecules such as proteins between CSF and brain interstitial space (Brightman & Reese, 1969; Fossan *et al.* 1985).
- (e) The embryonic CSF–brain barrier (Fig. 1E). This is a transient barrier between the CSF and brain parenchyma formed by strap junctions between adjacent neuroepithelial cells (Fig. 2Ea and b; Møllgård *et al.* 1987) which restrict all but the smallest lipid insoluble molecules from entering the brain (Fossan *et al.* 1985; Whish *et al.* 2015). They disappear progressively during development and are no longer present when this interface becomes ependyma (Fig. 2D; Møllgård *et al.* 1987).
- (f) The meningeal barrier (Fig. 1F). This consists of three distinct interfaces: (i) blood–arachnoid–outer CSF, (ii) blood–pia microvessel–outer CSF and



**Figure 1. Schematic diagram (middle right) of the five main barrier interfaces (A–D and F) in the adult and developing brain and an additional one present only in the embryo (E)**

The barrier-forming cellular layers at each interface are coloured green.

A, the blood–brain barrier is situated at the level of cerebral blood vessels (BV). Tight junctions (tj, arrowhead) are present between the endothelial cells (EC) restricting the paracellular cleft. AE, end feet from astroglial



cells; bm, basement membrane; PC, pericytes. Other important components of this interface are a basement membrane of extracellular matrix, within which are embedded pericytes (Engelhardt & Sorokin, 2009) surrounding the endothelial cells (Daneman *et al.* 2010b, Errede *et al.* 2014). Astroglial end feet encircle cerebral blood vessels during the first 2–3 weeks of postnatal development in rodents (Caley & Maxwell, 1970) although the encirclement appears to be less complete than previously thought (Korogod *et al.* 2015). The contribution of astrocytes to development and maintenance of barrier properties is controversial as will be discussed. These cellular structures are known collectively as the neurovascular unit (Neuwelt, 2004). *B*, the blood–CSF barrier is situated in the choroid plexus within each brain ventricle. Barrier-forming cells are the epithelial cells (CPE), which have tight junctions at their apical side (CSF facing, arrowheads). Blood vessels (BV) are fenestrated and do not form a barrier (arrows); apical microvilli increase exchange surface of epithelial cells to the internal CSF (i-CSF). *C*, circumventricular organs (including median eminence, pineal gland, area postrema, subfornical organ). Blood vessels have permeability characteristics similar to elsewhere in the body and have the functional property of allowing feedback penetration of peptide hormones controlled by the hypothalamic–pituitary axis. These peptides and other molecules are prevented from entering the CSF by tanycytes (TC), the specialized ependymal cells of these brain areas, connected by tight junctions between their apices (arrowhead); entry into the rest of the brain is prevented by tight junctions between astroglial cells (GC). Away from the tanycyte layer, ependymal cells lining the ventricular system are linked by gap junctions that allow free exchange between the CSF and brain interstitial fluid. *D*, ependyma in adult brain. Apart from areas where there are specialized tanycytes, ependymal cells are linked by gap junctions that do not restrict exchange of even large molecules, such as proteins, between CSF and interstitial space of brain (arrows). *E*, the embryonic CSF–brain barrier. In early brain development, strap junctions (arrowheads) are present between adjacent neuroepithelial cells (NE); these form a barrier restricting the movement of larger molecules, such as proteins, but not smaller molecules. *F*, the meningeal barrier is structurally the most complex of all the brain barriers. Barrier-forming cells are the outer layer of the arachnoid membrane (the arachnoid barrier cells; ABC); these have tight junctions (arrowheads) between adjacent cells forming a barrier between the outer cerebrospinal fluid (o-CSF) in the subarachnoid space (SAS) and more superficial dural layers (dural border cells (DBC) and the dura mater). Blood vessels (BV) in the SAS have tight junctions with similar barrier characteristics as cerebral blood vessels without surrounding pericytes and astrocytic end-feet. Blood vessels within the dura mater are fenestrated (f-BV). bm, basement membrane; gl, glia limitans. Redrawn from Saunders *et al.* (2016b).

(iii) outer CSF–brain surface interfaces (Fig. 2*F*). The outer CSF is the fluid-filled subarachnoid space. Tight junctions are present between the cells of the arachnoid barrier layer restricting permeability between fenestrated blood vessels in the dura and the subarachnoid space. The blood vessels within this space have tight junctions between the endothelial cells, but no astrocytic end feet or pericytes. This is the interface that restricts exchange between the blood and the outer CSF.

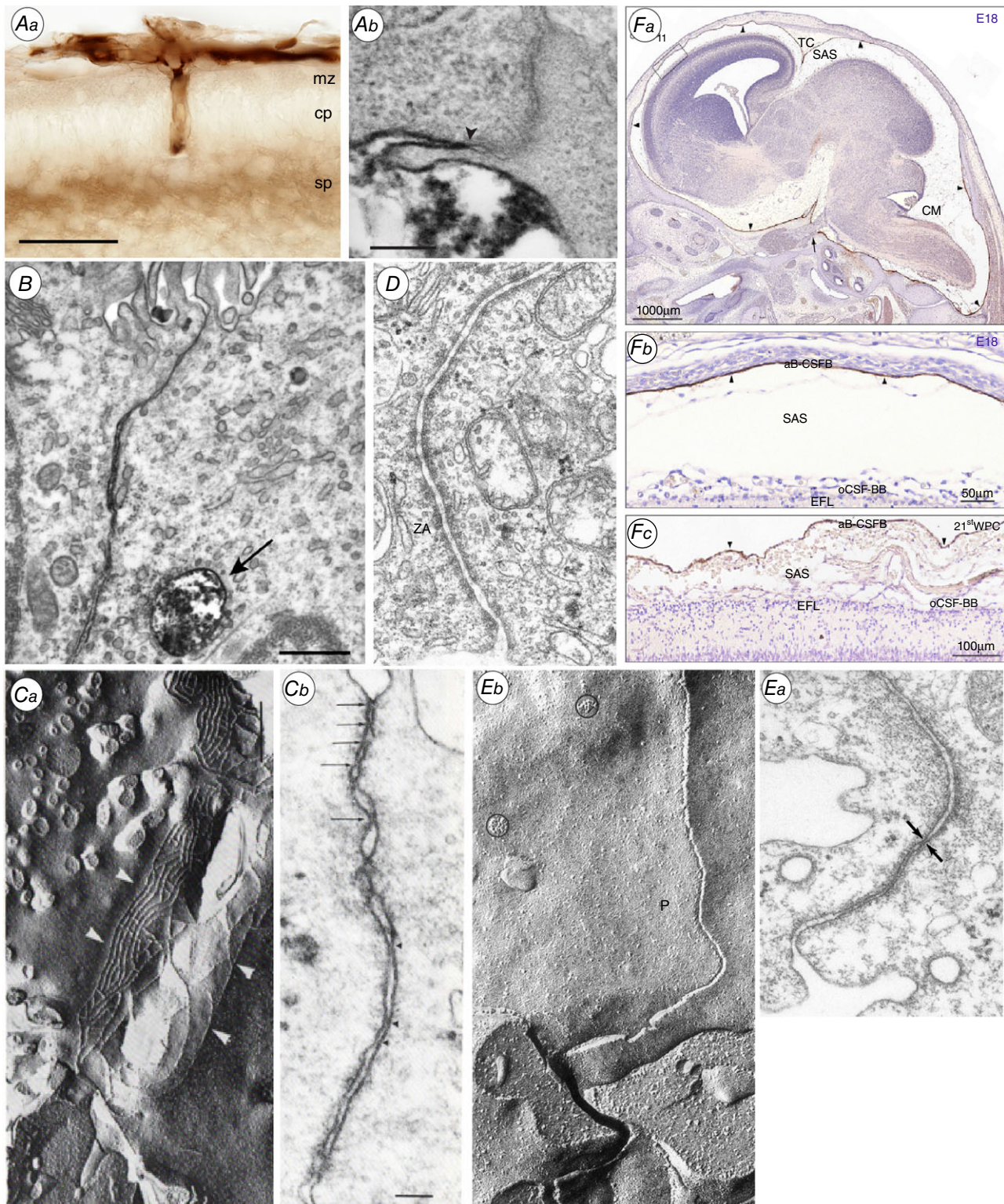
### Molecular structure of tight junctions in brain barrier interfaces

The molecular structure of tight junctions in brain barrier interfaces has been extensively studied both *in vivo* and *in vitro*. There are numerous proteins that interact to form intercellular junctional structures in many tissues including ZO-1, ZO-2, occludin and several claudins. However, there is some degree of specificity in the brain barriers. For example, in the mammalian blood–brain barrier claudin 5 expression is specific to this interface (Nitta *et al.* 2003; Kratzer *et al.* 2012) although in zebrafish it is apparently also expressed at the blood–CSF barrier (van Leewen *et al.* 2018). Angiogenesis and blood–brain barrier formation appear to be linked and occur simultaneously; by using

genetic mouse models, the effectors of Wnt/b-catenin signaling, including Lef1, Apccdd1 and tnfrsf19, have been shown to control these processes (reviewed in Daneman & Prat, 2015). Recently it has been shown that Reck (a glycosylphosphatidylinositol-anchored membrane protein) and Gpr124, an orphan G-protein-coupled receptor, promote both CNS angiogenesis and blood–brain barrier formation by activating the canonical Wnt7a/7b pathway (Cho *et al.* 2017).

### Functional implications of the junctional structures of brain barriers

It is important to stress that these interfaces are functionally much more than a structural barrier preventing passive exchange of molecules between the blood, CSF and brain. Nevertheless this seal is important not just because it prevents the intercellular passage of even very small (lipid insoluble) molecules, but also because this restriction allows the numerous cellular exchange mechanisms to act in concert over the full extent of the interface. For example without such a diffusion restriction it would not be possible for the ion exchange mechanisms to tightly control the ionic milieu of the brain's extracellular fluid that is essential for normal neuronal function.



**Figure 2. Morphology of the brain barriers illustrated in Fig. 1**

Aa, the blood–brain barrier. Light micrograph showing the localization of biotin ethylenediamine (BED) in the neocortex of opossum at P5 20–25 min after an intraperitoneal injection. Note that the staining for BED is most visible within the vessels, in the marginal and subplate zones. cp, cortical plate; mz, marginal zone; sp, subplate. Ab, localization of biotin–dextran (BDA3000) in the neocortex of P2 opossum 20–25 min after an intraperitoneal injection. Arrowhead points to site of the tight junction. Note that the marker is prevented from passing through the



intercellular space by the tight junction. From Ek *et al.* (2006). *B*, the blood–CSF barrier. Lateral ventricular choroid plexus (blood–CSF barrier) in P13 *Monodelphis*. Tight junction excludes entry of BDA3000 into CSF. Labelled dextran present in one large and several smaller endosomes. From Ek *et al.* (2003). *C*, Circumventricular organs. *a*, adjacent tanycytes facing the CSF in circumventricular organs are connected by an extensive network of apical tight junctional strands shown here by freeze fracture of the adult Mongolian gerbil subcommissural organ. *b*, a thin section electron micrograph of the same region in a neonatal animal shows multiple ‘kissing points’ (arrows) between neighbouring tanycytes indicating a complete occlusion of the paracellular pathway. From Madsen & Møllgård (1979). *D*, ependyma in adult brain. Junctional configuration in the ependymal layer of 125-day sheep fetus similar to those found in mature ependyma. ZA, zonulae adherens. Note unobstructed intercellular space. *E*, the embryonic CSF–brain barrier. *a*, thin-section electron micrograph of the neuroepithelial lining of the cerebral vesicle from an E19 sheep. The junctional zone exhibits very narrow intercellular clefts which at places seem to be totally occluded (arrows). This junctional configuration has been named ‘strap’ junction. *b*, characteristic freeze fracture single strand of strap junction perpendicular to CSF surface. From Møllgård *et al.* (1987). *F*, the meningeal barrier. Distribution of claudin-11 immunoreactivity in sagittal sections of E18 rat (*a* and *b*) and 21st wpc human (*c*) brain. Demonstrates a strong reactivity of the entire arachnoid barrier cell layer = arachnoid blood–CSF barrier (aB-CSFB, arrowheads). From Bröchner *et al.* (2015). CM, cisterna magna; EFL, radial glial end feet layer; SAS, subarachnoid space; TC, tentorium cerebelli.

### Alternative route into the brain

There have also been suggestions of ‘another route’ of entry into the very early embryonic brain before most of the characteristics of the six barrier interfaces have appeared. Bueno and colleagues have carried out extensive studies of the properties of CSF in chick embryos (eCSF) before the appearance of the choroid plexuses. They have also studied the transfer of proteins and other molecules between the blood and neural tube tissue (Parvas & Bueno, 2010; Bueno *et al.* 2014). Their evidence suggests that the internal environment of the CNS at this very early stage of development is already well controlled. The route of transfer appears to be in the brainstem lateral to the floor plate, in the ventral mesencephalon and in the most anterior part of the ventral prosencephalon. This location does not correspond to the site at which the choroid plexus later develops. From studies of zebrafish embryos, Lowery & Sive (2009) suggest that eCSF is a secretion of the neuroepithelium forming the neural tube. However, the studies of Bueno and colleagues demonstrate clearly that the composition of eCSF in the chick embryo depends importantly on transfer from the blood into eCSF (Parvas & Bueno, 2010; Bueno *et al.* 2014).

Lehtinen and colleagues (Zappaterra *et al.* 2007; Chau *et al.* 2015) have carried out extensive studies of the composition of amniotic fluid and eCSF in the period after neural tube closure (described below in the section ‘Proteins in fetal and newborn CSF’).

### Physiological and molecular evidence for effective barrier mechanisms in the developing brain

**Evidence for early establishment of ion gradients between CSF and plasma.** The identification of a gradient between CSF and plasma for even a single ion implies that at least two essential features of a barrier mechanism are present: (i) tight junctions between the cells of the

interface that are functionally effective in enabling the gradient to be established, and (ii) a cellular pump that sets up the gradient. This is amongst the most convincing evidence for functional barrier mechanisms in the developing brain. The ionic composition of CSF is thought to reflect that of the general internal environment of the developing brain, although there is some controversy about the extent to which CSF composition actually reflects blood–brain barrier properties; this is important for correct interpretation of CSF data in clinical conditions in adult patients (Lange, 2013) but does not seem to be used in neonates (see below). There have been numerous studies in a variety of species showing the presence of ion gradients between CSF and plasma, some from very early in development, as summarized in Table 1 and illustrated for the rat in Fig. 3.

A significant consideration in interpreting these results is that some ions ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) bind to proteins in plasma and CSF, particularly to albumin, and the protein concentration in fetal CSF is much higher at a time when it is lower in plasma (see below). Protein concentrations in CSF and plasma are important because they affect the proportion of each fluid that the electrolytes are dispersed in. Thus accurate comparisons between electrolyte concentrations in the two fluids can only be made when the units take account of this ( $\text{mEq kg}^{-1} \text{H}_2\text{O}$ ). As can be seen in Table 1, most estimates have used units based on volume of CSF or plasma (which includes protein) and thus need to be interpreted with caution. Nevertheless it is clear from Table 1 that concentration gradients are established early in brain development. No values appear to be available for human embryos or fetuses; values for neonates and infant are rare. No reference values are given in textbooks of fetal and neonatal medicine and CSF electrolytes do not appear to be routinely measured in neonates (Royal Children’s Hospital Melbourne; Royal Children’s Hospital, Brisbane; Prof. R. Ariagno, Neonatologist, Stanford University).



**Table 1. Electrolyte concentration (mmol L<sup>-1</sup> or mEq kg<sup>-1</sup> H<sub>2</sub>O) in CSF and plasma of embryos/newborns and adults**

Species	Age	Units	Fluid	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Ref
Monkey, term 168 days	E50–60	mEq kg <sup>-1</sup> H <sub>2</sub> O	CSF		3.6			3.8	1.85	1
			Plasma		4.1			4.9	1.5	1
	E90	mEq kg <sup>-1</sup> H <sub>2</sub> O	CSF		3.2			3.5	2.3	1
			Plasma		4.0			5.0	1.5	1
	E130	mEq kg <sup>-1</sup> H <sub>2</sub> O	CSF		2.9			2.9	2.2	1
			Plasma		3.8			4.9	1.5	1
	Adult	mEq kg <sup>-1</sup> H <sub>2</sub> O	CSF		2.6			2.3	1.9	1
			Plasma		4.5			4.6	1.2	1
	Rabbit, term 31 days	E23	CSF	142	4.1	113			1.5	2
			Plasma	143	6.5	104			3.2	2
		E27	CSF	140	4.0	110			1.6	2
			Plasma	147	6.0	108			3.6	2
		Adult	CSF	150	3.0	120			1.4	2
			Plasma	154	3.2	100			2.2	2
Rat, term 21 days	P0	mEq L <sup>-1</sup>	CSF	144	3.8	109				2
			Plasma	150	6.5	102				2
	P8	mEq L <sup>-1</sup>	CSF	144	3.8	107				2
			Plasma	166	7.1	98				2
	Adult	mEq L <sup>-1</sup>	CSF	152	3.0	122				2
			Plasma	153	5.0	106				2
Sheep, term 150 days	E44–50	mEq L <sup>-1</sup>	CSF	135	5.4	113		—	2.0	3
			Plasma	138	—	113		—	1.6	3
	E85–92	mEq L <sup>-1</sup>	CSF	144	3.5	123		3.4	1.9	3
			Plasma	136	3.8	104		6.05	1.8	3
	Adult	mEq L <sup>-1</sup>	CSF	148	3.1	128		2.45	1.8	3
			Plasma	138	3.4	115		3.4	1.4	3
Pig, term 115 days	5 cm CRL	mmol L <sup>-1</sup> H <sub>2</sub> O	CSF	126		115				4
			Plasma <sup>†</sup>	125		112				4
	6.5 cm CRL	mmol L <sup>-1</sup> H <sub>2</sub> O	CSF	120		115				4
			Plasma <sup>†</sup>	110		98				4
	12.5 cm CRL	mmol L <sup>-1</sup> H <sub>2</sub> O	CSF	125		122				4
			Plasma <sup>†</sup>	105		96				4
Pony, term 342 days	E312	mmol L <sup>-1</sup>	CSF	143	3.7	109				5
			Serum	134	5.7					5
	E342	mmol L <sup>-1</sup>	CSF	143	2.9	112				5
			Serum*	—	—	—				5
	Adult	mmol L <sup>-1</sup>	CSF	144	2.9	101				5
			Serum	132	4.7	94				5
Chick, hatch 20–21 days	E13	mEq L <sup>-1</sup>	CSF	121	3.9	104				6
			Plasma	117	6.3	101				6
	E15	mEq L <sup>-1</sup>	CSF	125	3.6	103				6
			Plasma	120	5.8	91				6
	E19	mEq L <sup>-1</sup>	CSF	129	3.8	109				6
			Plasma	134	7.8	93				6
	Adult	mmol kg <sup>-1</sup> H <sub>2</sub> O	CSF	159	4.2	142				7
			Plasma	160	4.7	115				7

(Continued)

This misses the possibility of using ion gradients between CSF and plasma as a means of assessing choroid plexus and blood–brain barrier function in human neonates, as suggested many years ago by Bito & Myers (1970): ‘Existence of normal cation concentration gradients between CSF and blood may serve as a criterion for the normality of the (foetal) blood–brain barrier’.

More recently transcriptomic studies have shown that key ion channel and transporter genes are expressed very early in embryonic life in the choroid plexuses (Liddelow *et al.* 2012, 2013). These are summarized in Fig. 4. One puzzle is the astonishing number of some channel genes that are expressed in development when we have no idea whether they are functionally effective.

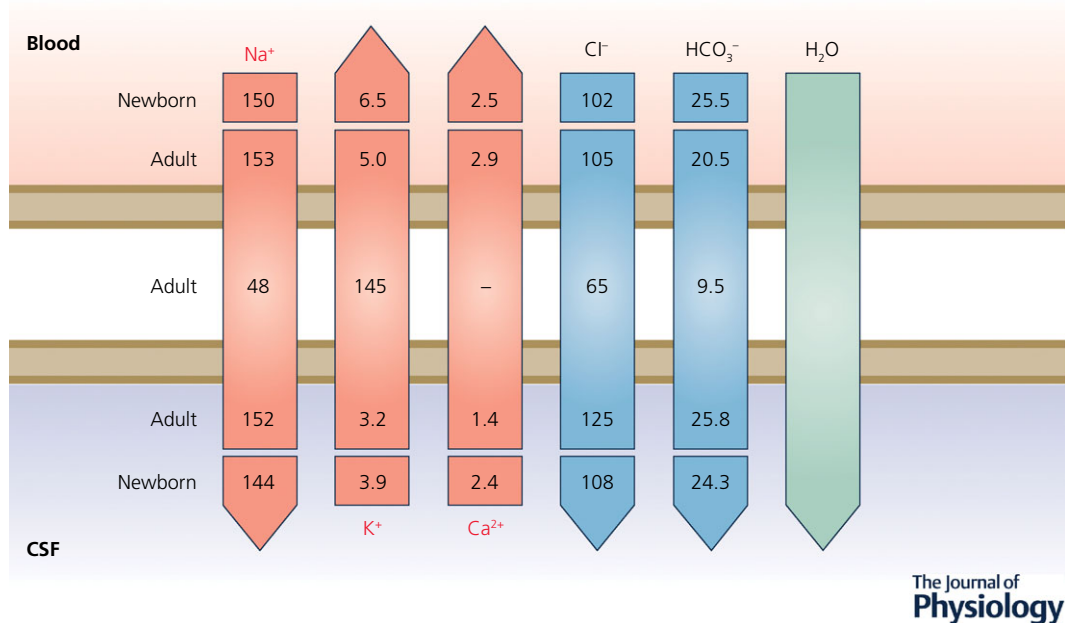
Table 1. Continued

Species	Age	Units	Fluid	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Ref
Human, 38 weeks' post-conception	P0–4 weeks	mmol L <sup>-1</sup>	CSF	138	3.2	117		1.6		8
			Plasma	136	5.4	105		2.3		8
	P7–12 months	mmol L <sup>-1</sup>	CSF	142	2.5	120		1.4		8
			Plasma	136	4.9	107		2.7		8
	P7–14 years	mm L <sup>-1</sup>	CSF	141	2.8	122		1.3		8
			Plasma	138	4.6	107		2.7		8
	Adult	mEq kg <sup>-1</sup> H <sub>2</sub> O	CSF	147	2.9	113	23	2.3	2.2	9
			Plasma	150	4.6	99	27	4.7	1.6	9

References: (1) Bito & Myers (1970), cisternal CSF; (2) Amtorp & Sørensen (1974), cisternal CSF; (3) Bradbury *et al.* (1972), cisternal CSF; (4) Flexner (1938), cisternal CSF; (5) Rosedale *et al.* (1982), atlanto-occipital subarachnoid CSF; (6) Stastny & Rychter (1976), ventricular CSF; (7) Anderson & Hazelwood (1969), cisternal CSF; (8) Heine *et al.* (1981), lumbar CSF; (9) Davson & Segal (1996), lumbar CSF. Note differences in units. The most appropriate are mmol L<sup>-1</sup> or mEq kg<sup>-1</sup> H<sub>2</sub>O as this allows for differences in protein concentration in CSF and plasma (see Davson, 1967, for discussion). These are markedly different early in development, as well as between CSF and plasma, see section on 'Proteins in CSF'. \*Not measured; †calculated from CSF/plasma ratios. CRL, crown rump length.

Many of these channels are expressed at a higher level in the developing rat choroid plexus than in the adult. Numerous gene family members for K<sup>+</sup> voltage gated channels were expressed at levels of between 2- and 210-fold higher in E15 choroid plexus than adult. Seven *Scn* genes (voltage gated sodium channels), nine *Trpv* channels, two chloride channel genes (*Clic1*, *Clic4*) and two cyclic nucleotide gated channels (*Cnga1* and *Cnga3*)

were also expressed at a higher level in the developing choroid plexus. In addition there were six ion channel genes that were expressed only at E15 (*Kcnmb2*, *Tmc5*, *Clnka*, *Scnn1g*, *Kcnh8*, *Kcnmb1*) and one in the adult (*Kcnk9*). Thirteen genes of the *Cacn* family of voltage gated Ca<sup>2+</sup> subunits were expressed at higher levels in E15 choroid plexus than in the adult (Liddelow *et al.* 2013).

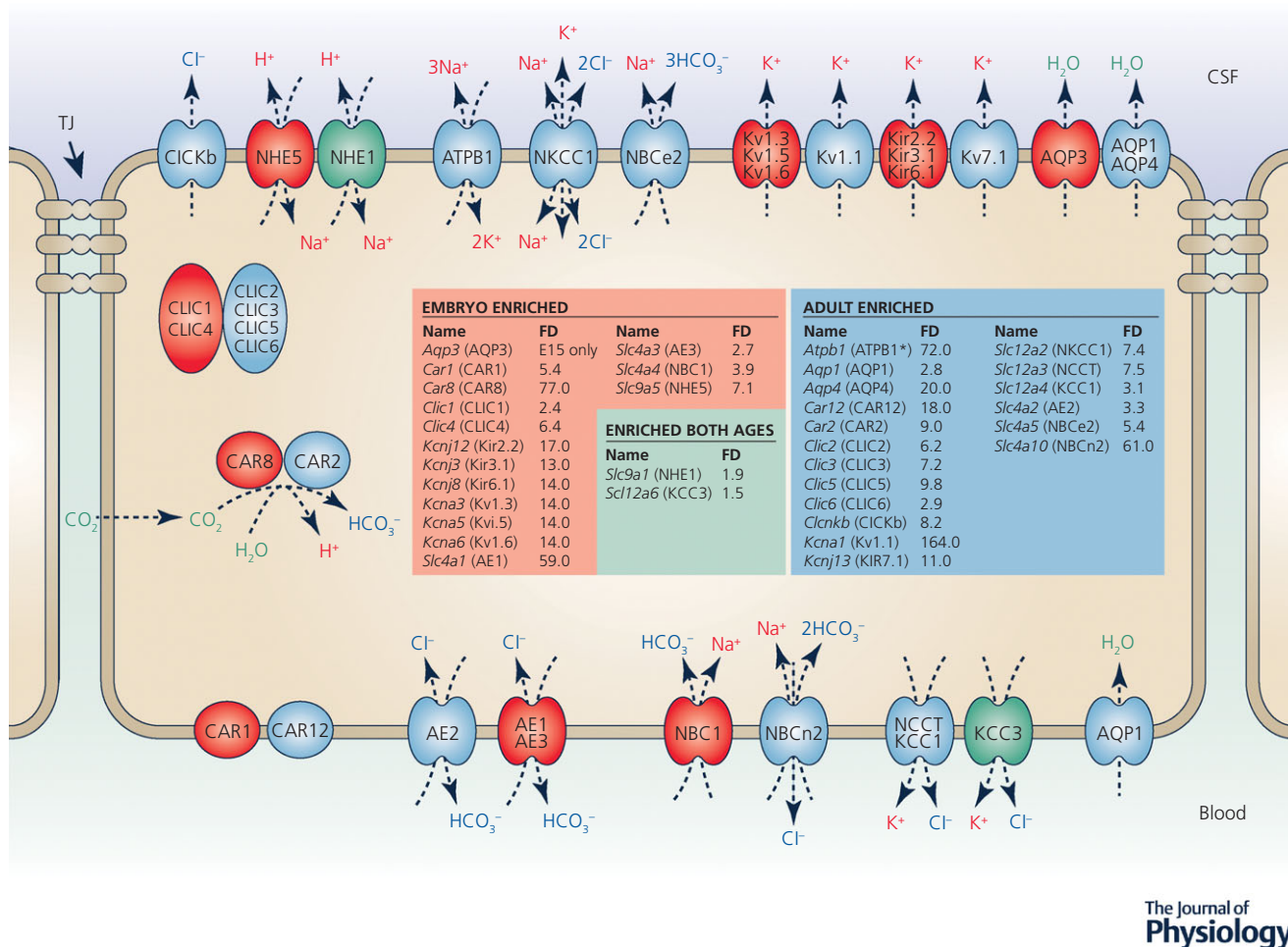


**Figure 3. Ion gradients between CSF and plasma in developing and adult rat brain**

A characteristic of CSF is its stable ionic composition that differs from that of plasma to an extent that cannot be explained by ultrafiltration, as was once thought. Data for CSF and plasma (mEq L<sup>-1</sup> H<sub>2</sub>O) are from Amtorp & Sørensen (1974) and for intracellular ions (mmol L<sup>-1</sup> H<sub>2</sub>O) from Fig. 8 in Johanson & Murphy (1990). The gradients are the consequence of the complex interactions between enzymes (notably carbonic anhydrase) ion transporters and ion channels, as illustrated in Fig. 4. The CSF secretion rate in the embryo and newborn is much lower than in the adult (Bass & Lundborg, 1973; Johanson & Woodbury, 1974), which is perhaps explained by the much lower expression of carbonic anhydrase and ATPases in the developing choroid plexus, as indicated in Fig. 4. Redrawn from Saunders *et al.* (2016b).

**Influx mechanisms.** The main functional groups of influx transporters in brain barrier interfaces defined from physiological studies are those for glucose (GLUT-1), amino acids (acidic, basic, neutral) monocarboxylic acids, peptides and ions, including metabolically important ions such as  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Mg}^{2+}$ . These mechanisms are summarized in Fig. 5. Most of the physiological studies were published many years ago, but this does not

diminish their value in understanding the importance of such mechanisms in the developing brain. There is a fundamental difficulty in designing adequate experiments to study the mechanisms that control the influx of these molecules and ions into the brain and CSF because it is important, but technically difficult, to distinguish between transport and metabolic incorporation into different brain compartments and structures. This problem was solved



**Figure 4. Localization of proteins for ion transporters, channels and associated enzymes and identification of their corresponding genes in adult and immature rat choroid plexus**

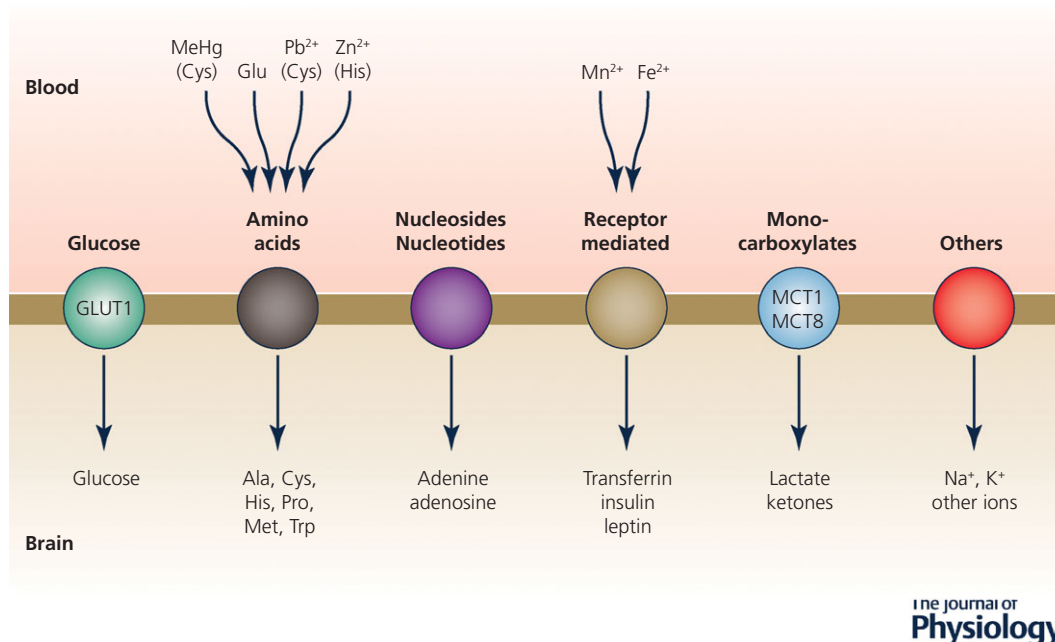
CSF secretion results from coordinated intracellular carbonic anhydrase activity and transport of ions and water from basolateral membrane to cytoplasm, then sequentially across apical membrane into the cerebral ventricles (Davson & Segal, 1996; Speake *et al.* 2001; Praetorius & Damkier, 2017). This process has only been studied in adult choroid plexus (Brown *et al.* 2004; Praetorius & Damkier, 2017). The membrane and intracellular locations of the ion channels, transporters and enzymes indicated are from Praetorius & Damkier (2017). Data from Liddelwell *et al.* (2013) compares expression of these genes and other functionally related genes in E15 and adult rat lateral ventricular choroid plexus. Blue indicates the genes that are upregulated (enriched) in the adult. Light red indicates genes that are expressed at a higher level at E15. We have assumed the same cellular/membrane location for members of the same gene family. The genes all had substantial but variable transcript numbers in the RNA-Seq analysis. In some cases where a gene was upregulated in the adult, the transcript number was also high in the embryo, suggesting this transporter or channel was likely to be functionally effective at both ages, e.g. the  $\text{K}^+$  channel *Kcnj13* (Kir7.1), *Slc12a2* (NKCC1) a  $\text{Na}^+$ - $\text{K}^+$ - $\text{Cl}^-$  exchanger and *Slc4a2* (NBCe2) a coupled  $\text{Na}^+$ - $\text{HCO}_3^-$  pump. *ATPB1* (*Atpb1b1*) is a  $\text{Na}^+$ / $\text{K}^+$ -ATPase. Green indicates genes that were expressed at similar levels at the two ages. There are many more channels that show age-related differential expression in choroid plexus, the functions of which are unclear. Redrawn from Liddelwell *et al.* (2016) with additional data from Liddelwell *et al.* (2013).



by Oldendorf (1971) with the introduction of a short pass technique initially for studies in adult animals. Although technically more difficult, it has also been applied to a limited extent and with some modifications in developing animals (Braun *et al.* 1980; Cornford *et al.* 1982; Lefauconnier & Trouvé, 1983). The essence of the technique is that a radiolabelled compound is injected as close as possible to the cerebral circulation and the experiment is rapidly terminated after enough time for only one circuit through the cerebral circulation. Usually two labelled compounds are compared: one, which would be expected to enter almost instantaneously, as a reference (e.g.  $^3\text{HOH}$ ), and a second that is the compound of interest, usually an amino acid or glucose. For those interested, we have reviewed these physiological experiments previously (Saunders *et al.* 2012, 2013).

**Amino acids.** It seems to have been assumed in most studies that entry into the brain was via the blood–brain barrier interface only and account was probably not taken of any entry via the choroid plexuses into the CSF. More importantly, in most studies it was not clear if the CSF and choroid plexuses had been removed prior to analysis of brain samples; any choroid plexus tissue or CSF included in the brain samples would have led to an overestimate of the contribution of blood–brain barrier transport of the amino acids into the brain, because at

least some amino acids accumulate in the choroid plexuses (al-Sarraf *et al.* 1997a) in addition to entering the CSF directly. There seems to have been only one series of studies of direct entry into CSF in the developing brain, which would mainly reflect entry across the blood–choroid plexus barrier (al-Sarraf *et al.* 1995, 1997b). The results showed greater entry of some amino acids into neonatal rat CSF than that of the adult and in some cases the entry into CSF was greater than into the brain; thus entry via the choroid plexus appears to be more important than across cerebral blood vessels at this early stage of brain development. The higher entry is presumably a reflection of the metabolic requirements of a rapidly growing brain, although some have interpreted it as due to ‘immaturity’ of the blood–brain barrier (e.g. Watson *et al.* 2006). There is only a small amount of information about amino acids in CSF in children, but this indicates that the CSF/plasma concentration ratios are higher in 0- to 3-year-old children than in older children for some amino acids (serine, valine, histidine and arginine, but lower for glutamate, Akiyama *et al.* 2014). Scholl-Burgi *et al.* (2008) and Jiménez *et al.* (2012) have published values for CSF and plasma amino acids in the first year of human life; they confirm that several amino acids are present in neonatal CSF at higher levels than later in life and with higher CSF/plasma ratios. This is consistent with the animal data showing higher transport early in brain development and is presumably a



**Figure 5. Influx transporters at the blood–brain barrier**

These are mainly SLC (solute carrier) transporters. See Hediger (2013) for comprehensive review. Only transporters for which there is physiological evidence for function are listed. As indicated in Table 2, transcripts for many more SLCs have been identified in molecular screens. Many of these genes are found in both endothelial cells of the blood–brain barrier and epithelial cells of the choroid plexuses. Others are unique to each interface as summarized in Table 2. Note, many metal ions that are potentially toxic can be carried in via some of these transporters.

reflection of metabolic requirements at a time when the brain is still growing rapidly.

There is now substantial information about gene expression in the blood vessels and choroid plexus of the developing brain. These data are summarized for SLC (solute carrier) transporters in developing mouse brain in Table 2. There is a strikingly large number of SLC transcripts in almost all families that have been identified in cerebral endothelial cells, choroid plexus epithelial cells or both. There thus appears to be a large amount of redundancy and which precise SLCs are responsible for specific amino acid or other molecules is unclear. However, there are instances where a mutation in a single *Slc* gene has serious effects on brain development; for example in mice deletion of *Slc7a5*, a large neutral amino acid transporter, leads to severe neurological abnormalities. In a few patients deleterious homozygous mutations of this gene were associated with motor delay and autistic traits (Tarlungeanu *et al.* 2016).

When many SLCs are involved in transport of the same amino acids it is difficult to assign specific SLCs to each amino acid class. In Table 3 we indicate *Slc* genes that may correspond to the transporters for molecules where there is evidence of their entry from blood into the developing brain, but many others may also be involved.

**Monocarboxylates.** A family of monocarboxylate transporters (MCTs) is involved in transport of monocarboxylates (e.g. pyruvate, lactate and ketone bodies) across plasma membranes, some of which are proton linked. These are now designated as members of the SLC16 family, of which there are 14 (Halestrup, 2013a). Thus far only four have been shown to be involved in monocarboxylate transport in humans (Halestrup, 2013b): SLC16A1 (MCT1), SLC16A3 (MCT4), SLC16A7 (MCT2) and SLC16A8 (MCT3). SLC16A1 (MCT1) is involved in transport of monocarboxylates across the endothelial cells of the blood–brain barrier (Halestrup, 2013a,b). *Slc16a1* (MCT1), *a2* (MCT8), *a6* (MCT7), *a8* (MCT3), *a9* (MCT9), *a12* (MCT12) and *a13* (MCT13) genes have been identified in adult mouse choroid plexus (Koehler-Stec *et al.* 1998; Marques *et al.* 2011; Saunders *et al.* 2015a). *Slc16a2* is a thyroid hormone transporter, which is expressed at similar levels in embryonic and adult choroid plexus; the others are all monocarboxylate transporters and expressed at a lower level in the rat embryonic plexus compared to the adult (Saunders *et al.* 2015a). Only *Slc16a10* (MCT10) is expressed at a higher level in mouse and rat embryonic choroid plexus compared to adult (Liddel *et al.* 2012; Saunders *et al.* 2015a). *Slc16a10* transports tyrosine, the amino acid precursor of the thyroid hormones tri- and tetraiodothyronine. The protein product of *Slc16a10* has been shown to have much stronger immunohistochemical staining in embryonic compared to adult choroid plexus (Saunders *et al.* 2015a).

This suggests that the very high expression of this transporter reflects an important role in thyroid hormone transport in early brain development.

Triiodothyronine (T3) and thyroxine (T4) are essential for normal brain development. Inadequate delivery of T4 to the developing brain is usually due to iodine deficiency; it may result in cretinism (Rivas & Naranjo, 2007; Skeaff, 2011). The choroid plexuses in the embryonic brain are prominent compared to vascularization of the rest of the brain and it has been suggested to be the main portal of entry into the developing brain (Johansson *et al.* 2008); this is consistent with high expression of *Slc16a10* (MCT10) in the choroid plexuses early in development. Transferrin (TTR) a thyroid hormone carrier highly expressed throughout development, is the major mechanism previously thought to deliver thyroxine to the brain in early stages of its development, whereas *Slc16a1*, which is the main thyroid hormone transporter expressed at the blood–brain interfaces in the adult, is expressed at only a low level in the developing brain (Kratzer *et al.* 2013). Notwithstanding that *Slc16a2* (MCT8) is expressed at similar levels in the developing and adult brain (in rodents), in humans mutations of this gene cause an X-linked syndrome of psychomotor retardation and altered thyroid hormone levels (López-Espíndola *et al.* 2014). Thus this gene is critical for normal human brain development. It seems not to be known what the expression level of *Slc16a10* (MCT10) is in the human fetus nor have mutations been reported. *Slc16a2* (MCT8) appears not to be critical for rodent brain development, as there is no neurological phenotype in knock-out animals (Visser, 2016). Thus there appear to be species differences in the relative importance of thyroid hormone transporters for brain development.

**Glucose.** GLUT-1 (SLC2a1, solute-linked carrier, SLC transporters) was the first glucose transporter to be described; its gene, *Slc2a1*, is expressed in both cerebral endothelial cells (Daneman *et al.* 2010a) and choroid plexus epithelial cells (Liddel *et al.* 2012, 2013). GLUT-1 is a facilitative transporter. It is the main member of this family in cerebral endothelial cells (Enerson & Drewes, 2006). *Slc2a3*, *a8*, *a12* and *a13* expression has also been identified in mouse cerebral endothelial cells (Table 2). *Slc2a1* is expressed in rodent choroid plexus (Table 2); in the embryo it is expressed at a slightly higher level (Liddel *et al.* 2013). Of the other glucose-transporting genes in this family that have been identified in rat choroid plexus (*Slc2a3*, *a4*, *a8*, *a12*, *a13* and *a15*, see Table 2) only *Slc2a12* is expressed at a level that is likely to be functionally significant (at five times higher in the adult, Liddel *et al.* 2013).

As summarized in Table 2, the SLC5 transporters are sodium–glucose co-transporters. *Slc5a1*, *a5* and *a6* have

Table 2. Comparison of *Slc* gene expression in developing mouse and rat brain endothelial cells and choroid plexus epithelial cells

Family	Transporter function	Choroid plexus epithelial cells	Present in both cell types	Cerebral endothelial cells
<i>Slc1</i>	High affinity glutamate and neutral AA transporter	—	<i>a3, a4, a5</i>	<i>a1, a4rg-ps, a2, a6</i>
<i>Slc2</i>	GLUT-1 transporter	—	<i>a1, a3, a4, a5, a6, a8, a10, a12, a13</i>	<i>a9</i>
<i>Slc3</i>	Heavy subunits of heterodimeric AA transporters	<i>ra1</i>		<i>a2</i>
<i>Slc4</i>	Bicarbonate transporter	<i>a1</i>	<i>a2, a3, a4, a5, a8, a10, a11</i>	<i>a7, a1ap</i>
<i>Slc5</i>	Na <sup>+</sup> /glucose co-transporter	<i>a1, ra8, ra10</i>	<i>a3, a5, a7, a6, a10</i>	<i>a3</i>
<i>Slc6</i>	Na <sup>+</sup> - and Cl <sup>-</sup> -dependent Na <sup>+</sup> /neurotransmitter symporters	<i>a11, a13, a14, a15, a20b</i>	<i>a1, a4, a6, a8, a9, a13, a15, a17, a20a</i>	<i>a2, a1, a7, a11, a20a</i>
<i>Slc7</i>	Cationic AA transporter/glycoprotein-associated	<i>a2, a7, a10, a11, ra3, ra12</i>	<i>a1, a5, a6, a7, a10, a11,</i>	<i>a3, a4, a8 a2, a14, a6os</i>
<i>Slc8</i>	Na <sup>+</sup> /Ca <sup>2+</sup> exchanger	—	<i>a1, a3</i>	<i>a2</i>
<i>Slc9</i>	Na <sup>+</sup> /H <sup>+</sup> exchanger	<i>ra3</i>	<i>a1, a2, a3r1, a3r2, a5, a6, a7, a8, a9</i>	—
<i>Slc10</i>	Sodium bile salt co-transport	—	<i>a3, a4, a6</i>	<i>a7</i>
<i>Slc11</i>	Proton coupled metal ion transport	—	<i>a2, a1</i>	—
<i>Slc12</i>	Electroneutral cation/Cl <sup>-</sup> co-transporter	<i>ra1, ra3</i>	<i>a2, a4, a6, a7, a8, a9</i>	<i>a5</i>
<i>Slc13</i>	Na <sub>2</sub> SO <sub>4</sub> /carboxylate co-transporter	—	<i>a4, a5</i>	<i>a3</i>
<i>Slc14</i>	Urea transporter	<i>a2.v1</i>	<i>a2</i>	—
<i>Slc15</i>	Proton oligopeptide co-transporter	—	<i>a2, a3</i>	<i>a4</i>
<i>Slc16</i>	Monocarboxylate transporter	<i>ma3</i>	<i>a1, a2, a3, a4, a6, a7, a8, a9, a10, a12, a13, a14</i>	<i>a11</i>
<i>Slc17</i>	Vesicular glutamate transporter	<i>a6, ra9</i>	<i>a5-8</i>	—
<i>Slc18</i>	Synaptic vesicular amine transporter	—	<i>a2</i>	<i>a3</i>
<i>Slc19</i>	Folate/thiamine transporter	—	<i>a1-3</i>	—
<i>Slc20</i>	Na <sup>+</sup> /PO <sub>4</sub> <sup>3-</sup> co-transporter	—	<i>a1, a2</i>	—
<i>Slc21</i>	Organic anion transporter	<i>m1a5, m5a1</i>	<i>1a4, 1c1, 2a1</i>	<i>2b1, 3a1, 4a1, 5a1</i>
<i>Slc22</i>	Organic cation/anion/zwitterion transporter	<i>a6, a23, ma17, ma21, ra2, ra7, ra9, ra18, ra25</i>	<i>a5, a17, a18, a8, a12, a15</i>	<i>a2, a3, a4, a21, a23,</i>
<i>Slc23</i>	Na <sup>+</sup> -dependent ascorbic acid transporter	<i>ra3</i>	<i>a2, ra1</i>	
<i>Slc24</i>	Na <sup>+</sup> /(Ca <sup>2+</sup> /K <sup>+</sup> ) exchanger	<i>a3</i>	<i>a4, a5, a6</i>	<i>a2</i>
<i>Slc25</i>	Mitochondrial carriers	<i>a18, a21, ra31-32, ra40, ra44, ra46</i>	<i>a1, a3, a4, a5, a10, a12, a14, a15, a16, a17, a20, a22, a24, a26, a27, a28, a29, a30, a32, a33, a35 a37, a38, a39, a45</i>	<i>a2, a11, a13, a18, a19, a23, a25, a34, a36, a40, a42, a44, a46, a47, a51, a53</i>

(Continued)



Table 2. Continued

Family	Transporter function	Choroid plexus epithelial cells	Present in both cell types	Cerebral endothelial cells
<i>Slc26</i>	Multifunctional anion exchanger	<i>a7, ra3-4</i>	<i>a2</i>	<i>a6, a1, a7, a10, a11</i>
<i>Slc27</i>	Fatty acid transport	<i>ma2, ma3</i>	<i>a1, a3, a4, a6</i>	<i>a4, a2</i>
<i>Slc28</i>	Na <sup>+</sup> -coupled nucleoside transport	<i>a3, ra2</i>	—	—
<i>Slc29</i>	Facilitative nucleoside transporter	<i>a2</i>	<i>a4, a2, ra3</i>	<i>a1</i>
<i>Slc30</i>	Zn <sup>2+</sup> efflux	<i>ma10, ra2</i>	<i>a3, a4, a5, a6, a9, ra10</i>	<i>a1, a7</i>
<i>Slc31</i>	Cu <sup>2+</sup> transporter	—	<i>a1, a2</i>	—
<i>Slc32</i>	Vesicular inhibitory amino acid transporter	—	<i>ra1</i>	—
<i>Slc33</i>	Acetyl-CoA transporter	<i>ma1</i>	<i>ra1</i>	—
<i>Slc34</i>	Sodium-dependent phosphate transport protein 2B	<i>ra2</i>	—	—
<i>Slc35</i>	Nucleoside-sugar transporter	<i>e2, f1, f3</i>	<i>a1, a2, a3, a4, d2, e4, f2, f5</i>	<i>a5, b1, b2, e1, e3</i>
<i>Slc36</i>	Proton-coupled AA transporter	—	<i>a1, a4</i>	—
<i>Slc37</i>	Sugar-phosphate/phosphate exchanger	—	<i>a1, a2</i>	<i>a3, a4</i>
<i>Slc38</i>	Sodium-coupled neutral AA transporter	<i>ma4, a11, ra1, ra7, ra8</i>	<i>a1, a3, a5, a4</i>	<i>a2, a6, a7, a9</i>
<i>Slc39</i>	Metal ion transporter	<i>ma4, ma12</i>	<i>a8, a10, a11, a14, a1, a3, a7, a12, a13</i>	<i>a6, a5, a9, a10, a14</i>
<i>Slc40</i>	Basolateral Fe <sup>2+</sup> transporter	—	<i>a1</i>	—
<i>Slc41</i>	Mg <sup>2+</sup> transporter	—	<i>a1, a2</i>	<i>a3</i>
<i>Slc43</i>	Na <sup>+</sup> -independent, AA transporter	<i>ma1, ma2</i>	<i>ra1, ra2, ra3</i>	—
<i>Slc44</i>	Choline-like transporter	<i>a3, ra4</i>	<i>a1</i>	<i>a2, a5</i>
<i>Slc45</i>	Sugar transporter	—	<i>a4, a1, a3</i>	<i>a2</i>
<i>Slc46</i>	Folate transporter	<i>a1</i>	<i>a3</i>	—
<i>Slc47</i>	Multidrug and toxin extrusion protein 1	<i>a1</i>	—	—
<i>Slc48</i>	Heme transporter	<i>a1</i>	—	<i>a1</i>
<i>Slc50</i>	Sugar efflux	<i>ma1</i>	—	<i>a1</i>
<i>Slc52</i>	Novel riboflavin transporter family	—	—	<i>a2</i>

Data from Affymetrix mouse genechip arrays for choroid plexus (E15, Liddelow *et al.* 2011a) and cerebral endothelial cells (Daneman *et al.* 2010a). Data from RNA-Seq Saunders *et al.* (2015a) rat E15 choroid plexus; Whish *et al.* (2015) E17 mouse ventricular zone (would have contained transcripts from endothelial and non endothelial cells). Transcripts that were present at both the blood-CSF and blood-brain barriers are shown in middle column. Transcripts that are present *only* in cerebral endothelial cells and not peripheral endothelial cells are underlined. AA, amino acid; m, mouse only; r, rat only.

**Table 3. Comparison of Slc transporter gene expression and function in embryonic mouse (E15) choroid plexus and embryonic mouse (E17) neuroepithelium**

Transporter	E15–adult mouse CP fold change	E17–adult mouse VZ fold change	Transport function	Reference
<i>Slc16a10</i>	66.8	1.2	Iodothyronines T3, T4	Porterfield & Hendrich (1992)
<i>Slc16a2</i>	n.d.	–2.4	Monocarboxylates	Lefauconnier & Trouvé (1983)
<i>Slc6a15</i>	11.4	–2.7	Neutral amino acids	
<i>Slc40a1*</i>	9.6	1.1	Fe <sup>2+</sup>	Morgan & Moos (2002)
<i>Slc7a11</i>	7.1	5.1	Cysteine, glutamate	Lefauconnier & Trouvé (1983)
<i>Slc4a1</i>	5.5	n.d.	Anion transporter (Cl <sup>–</sup> /HCO <sub>3</sub> <sup>–</sup> exchange)	Amtorp & Sørensen (1974), Damkier <i>et al.</i> (2010)
<i>Slc6a13</i>	4.6	2.4	GABA transporter	Al-Sarraf (2002)
<i>Slc1a4</i>	4.4	–1.6	Glutamate, neutral amino acids	Al-Sarraf <i>et al.</i> (1997a)
<i>Slc38a4</i>	4.2	0.94	Acidic and neutral amino acids	Lefauconnier & Trouvé (1983)
<i>Slc6a6</i>	4.1	0.90	Taurine	Al-Sarraf <i>et al.</i> (1997b)
<i>Slc4a4</i>	4.1	0.22	Na <sup>+</sup> –HCO <sub>3</sub> <sup>–</sup> cotransporter	Lefauconnier & Trouvé (1983)
<i>Slc7a1</i>	4.1	1.99	Acidic amino acids	Damkier <i>et al.</i> (2010)
<i>Slc39a8</i>	3.3	2.63	Zn <sup>2+</sup>	Lefauconnier & Trouvé (1983)
<i>Slc39a10</i>	2.8	n.d.	Zn <sup>2+</sup>	Chowanadisai <i>et al.</i> (2005)
<i>Slc25a37</i>	2.4	5.5	Fe <sup>2+</sup>	Chowanadisai <i>et al.</i> (2005)
<i>Slc14a2</i>	2.4	n.d.	Urea	Morgan & Moos (2002)
<i>Slc7a7</i>	2.3	n.d.	Dibasic and neutral amino acids	Johanson & Woodbury (1978)
<i>Slc39a11</i>	2.3	n.d.	Zn <sup>2+</sup>	Lefauconnier & Trouvé (1983)
<i>Slc43a2</i>	2.2	3.9	Large neutral amino acids	Chowanadisai <i>et al.</i> (2005)
<i>Slc1a3</i>	2.2	3.1	Glutamate	Oldendorf (1973)
				Al-Sarraf <i>et al.</i> (1997a)

Transcript fold change ratios compared to adult. References are for evidence of transport (blood to brain or CSF) in physiological experiments. Because of the large number of genes often involved in transport of similar classes of molecules, this is only an indication that genes located at these sites are functional. Data from Liddelow *et al.* (2012) and Whish *et al.* (2015). There are many more *Slc* genes that are expressed at a higher level in adult than in embryo, not listed here. \*Gene product ferroportin-1 identified in choroid plexus. n.d., not detected.

been identified in mouse cerebral endothelial cells and *Slc5a6* and *Slc5a10* are present in mouse choroid plexus.

There is good evidence from physiological experiments for glucose transport into the brain in both adult and neonatal rats and rabbits (Oldendorf, 1971; Cornford *et al.* 1982; Cornford & Cornford, 1986; Vannucci *et al.* 1994). Much of this is probably mediated by GLUT-1 (SLC2a1).

As indicated above with respect to amino acid transporters there is a general problem, yet to be resolved, where many transporters for the same substrate have evolved; there are currently no methods available for unequivocally linking a specific transporter to a specific contribution to transport. However, it is clear that at least in the case of GLUT-1 this apparent redundancy does not compensate for the

loss of GLUT-1 function (Ito *et al.* 2015). So perhaps the redundancy is there to entertain molecular biologists rather than having particular biological relevance.

A comprehensive study of gene expression profiles in embryonic mouse brain in bulk tissue samples and separated endothelial cells has recently been published (Hupe *et al.* 2017). Unfortunately its value is limited because the authors do not appear to have excluded choroid plexuses from their tissue samples and the method used to separate endothelial cells would have also included those from the choroid plexuses; these are considerably more vascularized in embryonic brain than is the brain itself (Johansson *et al.* 2008).

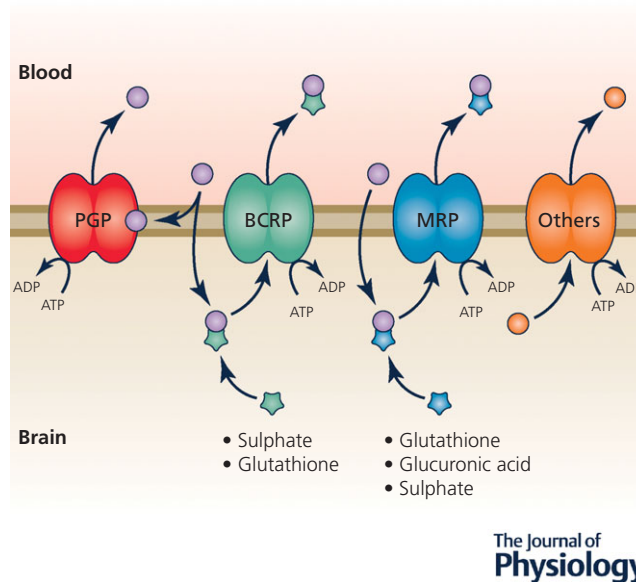
### Efflux mechanisms protecting the developing brain

The developing brain is protected by a combination of morphological features and cellular efflux mechanisms, as is the adult brain. An important practical and biological question is to what extent these are functional in the embryo, fetus and newborn. In particular how vulnerable is the developing brain to drugs and toxins reaching it from the mother across the placenta or via the milk? This will be the subject of a review to be published later in 2018 (Saunders *et al.*, *Annual Review of Pharmacology and Toxicology*); this aspect of brain barrier mechanisms will only be dealt with in outline here.

The main mechanisms that remove or exclude metabolic and potentially toxic compounds such as drugs across brain barriers in the adult brain are ATP-binding cassette (ABC) efflux transporters (Hartz & Bauer 2011) and some of the Slc families of transporters: Slc21 now re-designated as Slco1 (solute carrier organic anion transporter), Slc15 and Slc22 (Strazielle & Gherzi-Egea, 2015). The main efflux transporters at the adult blood–brain barrier are illustrated in Fig. 6. These are: ABCB1 (P-glycoprotein or MDR1) and ABCG2 (breast cancer resistance protein; BCRP). ABCC2 (multidrug resistance-associated protein 2; MRP2), ABCC4 (MRP4) have also been demonstrated at the blood–brain barrier interface (Strazielle & Gherzi-Egea, 2015). In cerebral capillary endothelial cells they export compounds into the blood. At the blood–CSF interface ABCC1 (multidrug resistance-associated protein 1; MRP1) is the main efflux transporter, but ABCC4 (MRP4) and ABCG2 (BCRP) are also present (Ek *et al.* 2010; Strazielle & Gherzi-Egea, 2015). They export compounds into the stroma of the plexus (Maliepaard *et al.* 2001; Ek *et al.* 2010). BCRP and MRP transport compounds that have been conjugated to specific transport motifs (glutathione, glucuronic acid or sulphated), which confer a wide range of substrate specificity and considerable overlap between transporters (see Fig. 6 and Löscher & Potschka, 2005). There are probably species differences in the level of expression

and functional capacity of these various efflux transporters. From limited studies of the developing brain it seems that expression changes with age during brain development at both interfaces (Schumacher & Møllgård, 1997; Virgintino *et al.* 2008; Ek *et al.* 2010; Daneman *et al.* 2010b; Kratzer *et al.* 2013); however, there are few functional studies dealing with the problem of whether these transporters are functionally effective. Even less is known about the presence of ABC transporters in brain barriers of the human embryo and fetus. We have published an immunohistochemical study of three of these transporters (P-glycoprotein, BCRP and MRP1) from 5 weeks' post-conception to mid-gestation (Møllgård *et al.* 2017) that reveals some striking differences between these transporters. Thus P-glycoprotein was not detectable at any barrier interface at 5 weeks' post-conception (but was detectable later in gestation), whereas the other two were detectable from the earliest ages but with differences in their distribution patterns (Møllgård *et al.* 2017).

The *Slc22* gene family of efflux transporters includes organic anion and cation transporters (OAT, OCT and OCTN proteins). *Slco* (SLC21) is a subfamily of organic anion transporting polypeptides (OATPs). The homology between rodent and human *Slco* genes is inexact. Judging from their cellular localization some may function as influx rather than efflux transporters (Strazielle & Gherzi-Egea, 2015). In rat choroid plexus most *Slco* and efflux *Slc* genes are expressed at similar or higher levels



**Figure 6. Efflux transporters at the blood–brain barrier**

These are mainly ATP-binding cassette (ABC) transporters. Some, e.g. P-glycoprotein (PGP; ABCB1), reduce entry into cells. Others, e.g. multidrug resistance protein (MRPs; ABCCs), ligand (drug or toxin) combines with glutathione, glucuronic acid or sulphate in cells before efflux. BCRP, breast cancer resistance protein (ABCG2). 'Others' include SLC efflux (SLCO) transporters.



than in the adult, except for *Slc22a8*, which is expressed at a lower level in the embryo and neonate. *Slc15a2* is well expressed in both brain and choroid plexus at similar levels in developing and adult choroid plexus. Comparison of the developmental profiles of ABC and SLC efflux transporters in rats shows the adult pattern is achieved earlier in the choroid plexus than in the blood–brain barrier (Strazielle & Ghersi-Egea, 2015). Nothing seems to be known about the expression or presence of SLC efflux transporters in the human fetal brain.

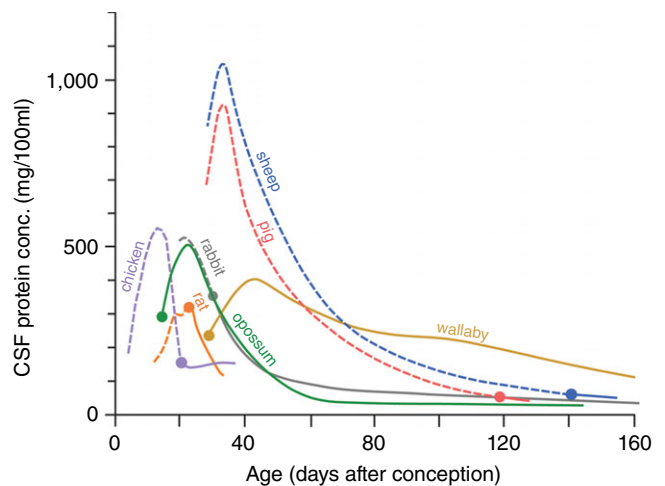
The extent to which gene or protein expression of an ABC transporter in the developing human brain corresponds to function *in vivo* cannot be determined experimentally. Thus animal studies are required, but very few studies of drug entry related to efflux transporter function in the developing brain have been performed; notable exceptions are the papers of Staud and colleagues (Staud *et al.* 2006; Cygalova *et al.* 2008). Knowledge of the presence and effectiveness of efflux transporters in the developing brain is essential for assessing the risk to fetuses when drugs are administered to a mother. Systematic understanding of efflux transporter expression and function would allow a rational approach to safer prescription of drugs in pregnant women.

### Proteins in fetal and newborn CSF

The protein concentration in fetal CSF is much higher than in the adult (Dziegielewska & Saunders, 1988; Saunders *et al.* 1999). The first quantitative estimates of protein concentration in CSF of embryos appear to be those of Klosowski (1963) in embryonic and postnatal cats. He used an uncalibrated spectrophotometric method and reported that the protein concentration in 10 cm (E48, term is E63–70, Evans & Sack, 1973) embryos was 21.6 times higher than in the adult. Wahle *et al.* (2014) recorded a median value of 10 mg (100 mL)<sup>−1</sup> for adult cat, which if assumed for Klosowski's data gives 216 mg (100 mL)<sup>−1</sup> at a post-conception age of about 60 days (cf. Fig. 7). This is somewhat below the values for sheep and pig fetuses at this age but much higher than species with smaller brains (Fig. 7). It has been suggested that a possible function of the high concentration of protein in CSF of the developing brain is to provide an oncotic pressure gradient, which expands the ventricular system (Saunders, 1992). Deflation of the ventricular system in the chick embryo resulted in very abnormal brain development (Desmond & Jacobsen, 1977). Thus a higher concentration of protein in the ventricular system of larger brains may be a component of the mechanisms required for achieving the larger brain, which essentially grows around the expanding ventricles. The presence of strap junctions between cells of the neuroepithelium that line the ventricular system are also likely to be an important

structural component of this mechanism. They have been shown to be impermeable to proteins and other large molecules early in brain development (Fossan *et al.* 1985; Whish *et al.* 2015). Without such a diffusion impediment, the oncotic pressure generated by proteins in CSF would be ineffective. The peak in CSF protein in the various species studied (Fig. 7) coincides with the time when the developing brain is generating the neurons that form the cortical plate, the forerunner of the neocortical layers that form the neocortex (see Table 1 in Dziegielewska *et al.* 2000). The diffusion restriction provided by the strap junctions of the neuroepithelium may provide an important mechanism for regulation of the local environment to which the cells of the neuroepithelium are exposed.

The protein composition of CSF in the developing brains has been reported for a number of species (reviewed in Dziegielewska & Saunders, 1988). These studies used immunological methods to characterize and quantify major proteins in CSF from developing brains. More recently proteomic studies of the composition of CSF at very early stages of brain development, including before the differentiation of the choroid plexuses, have been published (chick and rat embryos, Parada *et al.* 2005; rat and human embryos, Zappaterra *et al.* 2007). These comprehensive methods identified many more proteins in CSF from developing brain in these species. In a



**Figure 7. Total protein concentration in CSF of various species at different post-conceptual ages**

Ordinate: total protein concentration mg (100 mL CSF)<sup>−1</sup>. Abscissa: post-conceptual age in days. Filled circles indicate time of birth. Data for sheep from Dziegielewska *et al.* (1980a); pig (Cavanagh *et al.* 1982); rat (Dziegielewska *et al.* 1981; Chciu *et al.* 1984); tammar wallaby, *Macropus eugenii* (Dziegielewska *et al.* 1986); opossum, *Monodelphis domestica* (Dziegielewska *et al.* 1989); chick, (Birge *et al.* 1974); rabbit (Ramey & Birge (1979). Adult values not shown. For mammalian species, including human (see Davson, 1967) and marsupials mean values are between 23 and 31 mg (100 mL)<sup>−1</sup>. Chicken is 141 mg (100 mL)<sup>−1</sup>.

comparison of amniotic fluid and early CSF from mouse embryos, Chau *et al.* (2015) showed that amniotic fluid at E8.5, the time of neural tube closure, contained many more proteins (764) than eCSF at E10.5 (504) or E14.5 (410). In the initial stages of development after neural tube closure there is a decline in the total protein concentration in eCSF, followed by an increase around the time of differentiation of the choroid plexuses as previously shown (Dziegielewska *et al.* 1981). These studies of eCSF coupled with ones that begin to relate specific properties of CSF to particular features of early brain development (e.g. Gato & Desmond, 2009; Lehtinen & Walsh, 2011; Lehtinen *et al.* 2011, 2013; Gato *et al.* 2014) are opening a new era of understanding of blood–brain and blood–CSF barrier properties and how they relate to brain development.

There is much less information about proteins in human fetal CSF. Adinolfi and colleagues have published some valuable data on this and have shown that as in animal species the total protein concentration is very high compared to the neonate and adult. Thus at 14 weeks' gestation the CSF total protein concentration was about  $120 \text{ mg (100 mL)}^{-1}$  increasing to over  $560 \text{ mg (100 mL)}^{-1}$  at 20–24 weeks' gestation (Adinolfi & Haddad, 1977). The dominant protein was albumin with a substantial contribution from  $\alpha$ -fetoprotein. However, as the samples were from aborted fetuses it is uncertain whether the state of the fetuses would have affected the protein content of the CSF sampled. In the newborn period CSF is sampled in investigation of a number of pathological conditions, but some normal values have been published; the level in preterm infants is higher than at term and than in adults (see Table 1 in Saunders, 1977; Bonadio, 1992; Srinivasan *et al.* 2012). It has been suggested that the higher CSF protein concentration in neonates reflects increased permeability of the blood–brain barrier (Bonadio, 1992; Srinivasan *et al.* 2012). CSF protein concentration does not reach adult values until about 6 months after birth (Adinolfi, 1985), which has been taken by some as the age when the blood–brain barrier is 'mature' (Rodier, 1995; Watson *et al.* 2006). In reality the concentration of protein in neonatal CSF is but one of many indicators of blood–brain or more accurately blood–CSF barrier function. Rather than indication of a more permeable blood–brain barrier a more critical determinant is likely to be the turnover of CSF, which is less in the developing brain.

Adinolfi & Haddad (1977) and Adinolfi (1985) interpreted the high concentration of protein in human fetal CSF as evidence that 'permeability of the blood–CSF barrier is 'incomplete' or 'immature' in humans; from permeability experiments with  $^{125}\text{I}$ -labelled proteins in newborn rats they drew the same conclusion (Adinolfi & Haddad, 1977). From measurements of total protein concentration in fetal and newborn rabbits Ramey & Birge (1979) concluded that 'the blood–cerebrospinal fluid barrier to proteins begins to function by 18 to 20 days of

gestation'. From their data on total protein concentration in CSF of chick embryos, Birge *et al.* (1974) concluded 'a specialized restrictive barrier to protein does not operate between plasma and CSF in chick embryos of 5–10 days.' However, Ramey & Birge 1979 make the important point that because the turnover of CSF in the developing brain is much less than in the adult (Bass & Lundborg, 1973; Johanson & Woodbury, 1974), this could account for the higher level of protein in CSF.

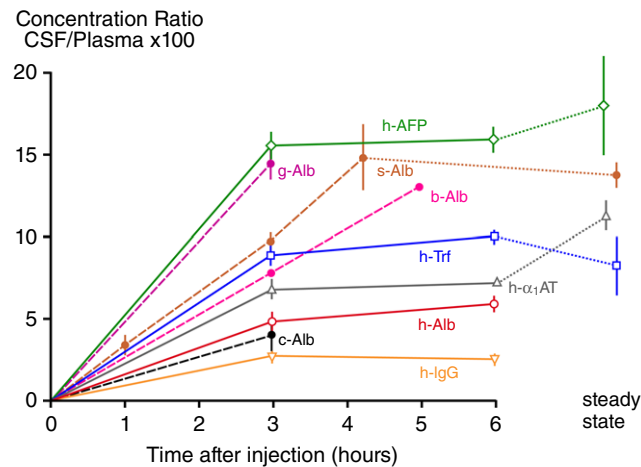
#### **Transfer of plasma proteins across choroid plexus epithelial cells.**

The levels of proteins in fetal and newborn CSF may be set by the turnover of CSF, but there is good experimental evidence that their entry into CSF from plasma is a result of specific transcellular transfer of plasma proteins across choroid plexus epithelial cells (Dziegielewska *et al.* 1980b, 1991; Habgood *et al.* 1992; Knott *et al.* 1997; Liddelow *et al.* 2009, 2011b). Johansson *et al.* (2008) have suggested that it may be the total amount of protein in CSF that is important as a reflection of transport capacity rather than the concentration of protein. Counts have been made of the number of cells in choroid plexuses immunostained for individual proteins, e.g. albumin,  $\alpha$ -fetoprotein and transferrin, at different stages of brain development in different species (Jacobsen *et al.* 1982a,b, 1983; Liddelow *et al.* 2009). The proteins derive from plasma and cross the plexus epithelial cells by what appears to be a specific transfer mechanism. This has best been studied extensively for albumin (Dziegielewska *et al.* 1980b, 1991; Habgood *et al.* 1992; Knott *et al.* 1997; Liddelow *et al.* 2009, 2011b). Depending upon the animal species, different choroid plexuses can distinguish different species of albumin (Dziegielewska *et al.* 1980b, 1991; Habgood *et al.* 1992; Knott *et al.* 1997). Thus fetal sheep choroid plexus distinguishes between on the one hand its own albumin and bovine albumin compared to human albumin, which is transferred into CSF to only about one third of the other albumins (Dziegielewska *et al.* 1980b, 1991) as illustrated in Fig. 8. The proportion of cells that are immunostained for individual proteins is much higher in species with large brains and ventricular systems. For example in human fetuses about 35–40% of the plexus cells in the lateral and third ventricle are positive for albumin and this declines to about 10% at birth (Jacobsen *et al.* 1982a). In fetal sheep at E30–40, 40–50% of cells in the lateral ventricle are immunopositive for albumin, this falls to about 1% in late gestation (Jacobsen *et al.* 1983) when the brain is more mature than in human fetuses. In *Monodelphis domestica* at P1 when the lateral ventricular choroid plexus first appears, < 1% of cells are positive for albumin. This increases to about 3% in young adults. We have not systematically counted albumin positive cells in rodents, but it is clear in our material that the proportion of these cells is small in rats and mice. For total plasma

proteins (to which albumin contributes only a fraction) in rat embryos at E13–16, some 14–17% of cells were protein positive and this declined to 10% by E18. The ventricular system in rodents is larger than in *Monodelphis*.

A screen of transcriptomic data from mouse choroid plexus, confirmed by single cell PCR, identified several albumin binding molecules which were then shown to be localized in choroid plexus epithelial cells: glycophorin A (GYPA) and SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin/BM-40/culture-shock protein). These albumin binding molecules might be involved in transcellular transport of albumin in the choroid plexus (Liddelow *et al.* 2012). By using an *in situ* proximity ligation assay (Duolink©) we have been able to show intracellular co-localization of mouse albumin and Sparc in choroid plexus cells of post-

natal mice (Fig. 9); there was no such co-localization for injected human albumin confirming the species specificity of the co-localization (Liddelow *et al.* 2014). An additional step in the process may be involvement of vesicle-associated membrane proteins, which are members of a family of SNARE proteins (soluble NSF attachment protein receptors), mostly involved in vesicle fusion. The genes for three of these vesicle-associated membrane proteins (*vamp1*, *vamp5* and *vamp8*) were identified in the transcriptomic study of mouse choroid plexus and the localization in these cells demonstrated by immunohistochemistry (Liddelow *et al.* 2012). The ultrastructure of a potential transcellular route for albumin in fetal choroid plexus cells was previously demonstrated using a combination of transmission electron microscopy (EM), high voltage EM and gold labelling EM immunocytochemistry; it has been designated as a system of tubulocisternal endoplasmic reticulum (Møllgård & Saunders, 1975, 1977; Balslev *et al.* 1997a). Figure 10 and Fig. 11 illustrate how these various components of a transcellular albumin transfer system might operate.



**Figure 8. Penetration of human plasma proteins from blood into CSF of 60-day sheep fetuses**

Human plasma was injected intravenously and blood was sampled to give an estimate of mean plasma concentration. At the times indicated CSF was sampled from cisterna magna. Concentrations of individual proteins in CSF and plasma were estimated by radial immunodiffusion assay. Abscissa: time in hours following i.v. injection; ordinate: CSF concentration/plasma concentration  $\times 100$ . Steady state indicates CSF/plasma ratio for naturally occurring sheep proteins. Mean  $\pm$  SEM for three to six experiments. All injected proteins were human (h-) except for s-Alb ( $^{35}$ S-sheep albumin) g-Alb (goat albumin) and b-Alb (bovine albumin) measured using sheep anti-goat or anti-bovine albumin antiserum. Experimental details and data are from Dziegielewska *et al.* (1980a, 1980b, 1991).  $\alpha_1$ AT,  $\alpha_1$ -antitrypsin; AFP,  $\alpha$ -fetoprotein; Alb, albumin; IgG, immunoglobulins; Trf, transferrin. The sheep fetus does not possess any IgG of its own, and hence no steady state ratio is shown. Note (i) there is an apparent relation between molecular size and permeability (the largest molecule, IgG, has the lowest ratio and the smallest molecule, AFP, has the largest ratio; however, all of these ratios except for IgG are higher than would be expected from passive diffusion (Saunders, 1992); and (ii) albumin from different species may have different ratios, which suggests that there is a selective mechanism that transports proteins from plasma to CSF. The route of protein transfer appears to be via the epithelial cells of the choroid plexus (Jacobsen *et al.* 1983; Dziegielewska *et al.* 1991).

### Misconceptions and controversies

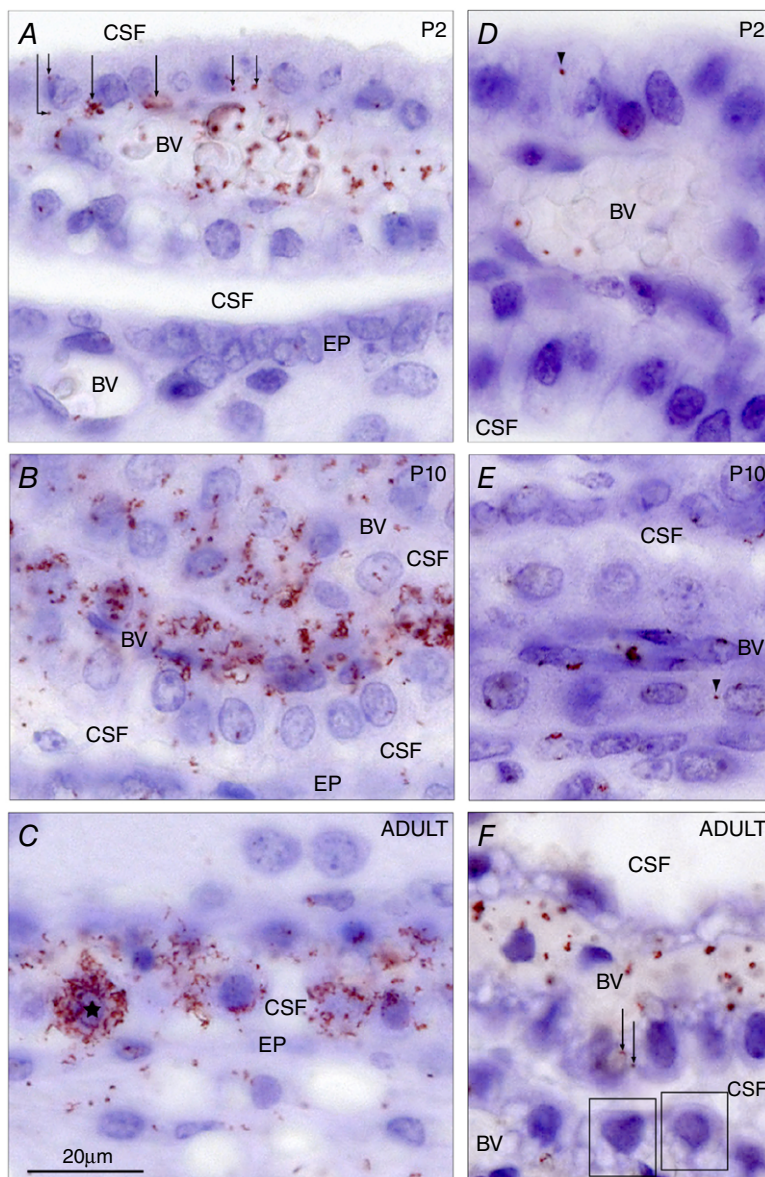
**Continued use of the term ‘immature’ to indicate a functional deficiency in brain barrier mechanisms.** Examples of continued use of the term ‘immature’ to indicate a functional deficiency in brain barrier mechanisms can be found in Allen (2015), Oberdick *et al.* (2016), Panfoli *et al.* (2016) and Amaraneni *et al.* (2017). This usually turns out to be a consequence of poor experimentation or an assumption that because a mechanism is different from that in the adult it is in some way deficient. In our view such differences are more likely to reflect an important match between appropriate barrier function and particular features of the stage of brain development. This concept has the merit of suggesting a way forward in future research whereas dismissing something as ‘immature’ is a dead end for both understanding and future enquiry. It seems reasonable to write in terms of maturation of blood–brain barrier mechanisms when comparing the embryo and adult (Hupe *et al.* 2017), but this should not be taken to imply deficient function. That needs to be studied in the context of the relevant aspects of brain development in different brain regions. At present there is not much information on this approach as it has rarely been examined (but see above section on ‘Proteins in fetal and newborn CSF’ and also below).

**Do the first blood vessels that grow into the brain anlage have functionally effective tight junctions?. Or is there ‘tightening’ of the blood–brain barrier as**



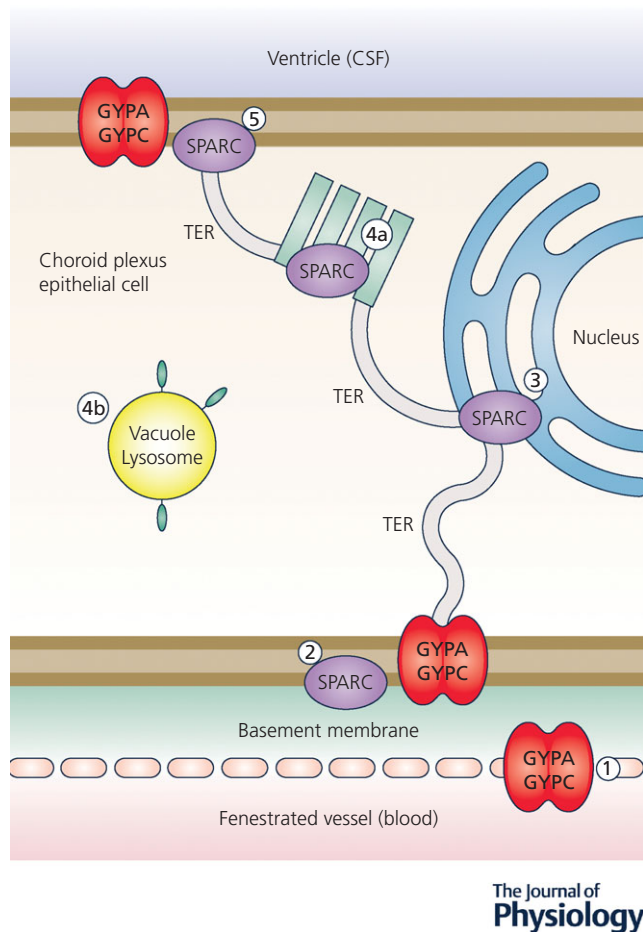
brain development proceeds (e.g. Cullen *et al.* 2011)? Early developing cerebral blood vessels may not be as fully developed in their molecular and morphological characteristics compared to the adult as they are operating at much lower blood pressures. This might influence the molecular composition of junctional complexes but does not mean that their permeability properties are different. Ek *et al.* (2006) have shown that biotin ethylenediamine (molecular mass 286 Da) and 3000 Da biotin dextran are excluded from entering the neocortex of *Monodelphis domestica* at P5 (a stage when blood vessels are first entering the neocortex) by functionally effective tight junctions. Umans *et al.* (2017) have shown that in the CNS of zebrafish angiogenesis and blood–brain barrier characteristics (barriergensis) occur simultaneously as the first blood vessels grow into the neural tissue. They

carried out some ingenious experiments *in vivo* in which they compared the first appearance of the glucose transporter Glut-1 and an angiogenesis marker, plvap:EGFP (plasmalemma vesicle-associated protein: enhanced green fluorescent protein) in a double transgenic fish. Glut-1 is an important early characteristic of cerebral endothelial cells, but its presence in itself does not tell anything about the permeability of the vessels. This could be investigated by immunostaining for a protein in plasma, as a test of large molecule permeability or injection of a labelled small molecule such as biotin ethylenediamine. Fleming *et al.* (2013) have published permeability experiments and EM observations at very early stages of zebrafish development. They claim that Evans blue entered the brain at 3 days post-fertilization (dpf) but not at 5 dpf or subsequently; exclusion from the brain of sodium fluorescein



**Figure 9. Cellular distribution of mouse albumin-SPARC (A–C) and human albumin-SPARC (D–F)**

Demonstrated by *in situ* Proximity Ligation Assay (*in situ* PLA) signals in the lateral ventricular choroid plexus at P2 (A and D), P10 (B and E) and adult (C and F). Note at P2 that most of the signal was distributed within blood vessels (BV), often associated with red blood cells. Under this magnification it is possible to distinguish positive signals distributed in the basolateral cytoplasm of choroid plexus epithelial cells (arrows). In contrast to the mouse albumin-SPARC signal, the human albumin-SPARC signal (D) was very rarely found and nearly always only associated with blood vessels (BV). Only one positive signal was found and it appears to be located in the extended extracellular space (arrowhead). At P10 (B and E) a very strong signal was visible for mouse albumin-SPARC (B) in many plexus cells distributed throughout the whole cytoplasm, blood vessels (BV) and also in the CSF. The human albumin-SPARC signal (E) was generally only present in blood vessels (BV) but a very occasional signal was detected in the apparent extended extracellular space (arrowhead). The CSF space was negative. In the adult (C and F) a mouse albumin-SPARC signal was distributed clearly throughout the cytoplasm of some choroid plexus epithelial cells (one cell marked with an asterisk). The positive signal was also detected in the ependymal (EP) and subependymal layers of the brain. The human albumin-SPARC signal (F) was visible in blood vessels (BV) but not in the CSF and only very sporadically in the plexus epithelium (two positive red dots are indicated by arrows). Otherwise plexus epithelial cells (boxes) showed no *in situ* PLA signal. BV, blood vessels; CSF, cerebrospinal fluid; EP, ependymal. Same magnification, scale bar is 20  $\mu$ m. From Liddelow *et al.* (2014).



**Figure 10. Proposed transepithelial pathway for albumin transfer through choroid plexus epithelial cells**

Cartoon of suggested routes of albumin transfer from plasma into CSF across the choroid plexus epithelium. GYPA/C in the endothelial cells may deliver albumin to the basement membrane (1) from where it can be taken up into the plexus epithelium by GYPA/C or SPARC (2). From here albumin may travel along a SPARC-specific pathway through the tubulocisternal endoplasmic reticulum (3) (see Fig. 11) and Golgi (4a), or via a VAMP-mediated pathway in vacuoles, lysosomes or multivesicular bodies (4b). On the apical surface of the plexus epithelium, GYPA/C may be involved in efflux of protein from the cell into the CSF of the ventricles (5), as validated by extensive GYPA immunoreactivity in embryonic plexus (Liddelov *et al.* 2012). In the adult, the lack of immunoreactivity in the endoplasmic reticulum and Golgi (Liddelov *et al.* 2012) along with increased expression of gene products for VAMP molecules suggests that the majority of transport possibly occurs via VAMP-mediated vesicular/lysosomal transport such as shown in (4b). CSF, cerebrospinal fluid; GYPA, glycophorin A; GYPC, glycophorin C; SPARC, secreted protein acidic and rich in cysteine; VAMP, vesicle-associated membrane proteins. Redrawn from Liddelov *et al.* (2012).

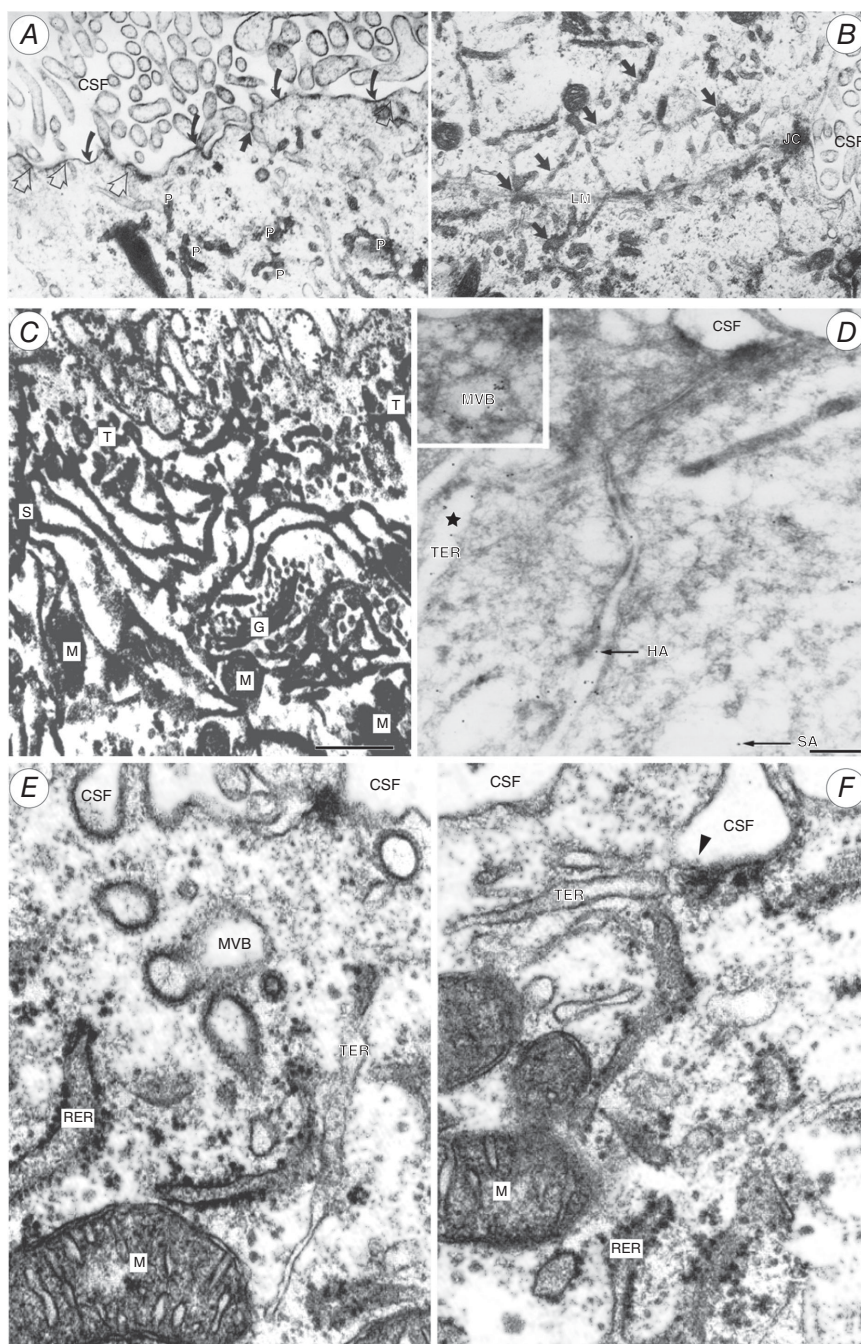
was not observed until 10 dpf. The images provided are unconvincing. Evans blue is an unsuitable marker for blood–brain barrier studies (Saunders *et al.* 2015b); the sodium fluorescein solution used (10%) is strongly hyperosmolar and therefore likely to be damaging to fragile blood vessels. The EM evidence of tight junctions was either ‘not illustrated’ or unconvincing in poor quality micrographs. Perhaps no evidence is better than poor quality evidence.

Van Leewen *et al.* (2018) have used a similar approach to Umans *et al.* (2017) and report that the tight junction protein claudin 5 is present in both cerebral endothelial cells and choroid plexus epithelial cells. In developing rodent choroid plexuses the only expression and immunostaining for claudin 5 is in blood vessels; in rodents claudin 5 is regarded as specific for the blood–brain barrier (Kratzer *et al.* 2012). Van Leewen *et al.* (2018) provide additional evidence for the proposal that in early development vascularization of the choroid plexuses compared to that of the brain indicates that the plexuses are likely to be a more important route of entry into the brain early on (Johansson *et al.* 2008).

Ben-Zvi *et al.* (2014) conducted experiments in mice at E13.5, E14.5 and E15.5 in which 10 kDa dextran marker was injected into the liver of the embryos. In the youngest embryos dextran was identified in cells in the CNS parenchyma and it was suggested that this had crossed the cerebral vessel walls, thus indicating that the vessels were permeable. It was not clear whether the route was intercellular or transcellular. An alternative explanation is that the dextran may have been transferred across the choroid plexus cells into CSF and taken up from there into parenchymal cells. At E14.5 it was reported that there was a small amount of dextran in the interstitial space around some vessels, although it was not apparent in the micrographs provided. At E15.5 the dextran was found confined to the cerebral blood vessels and on this basis it was suggested that the blood–brain barrier in the mouse becomes ‘functional’ at E15.5. It would require ultrastructural studies with a suitable small marker to confirm this (cf. Ek *et al.* 2003). At 5 weeks post-conception in human embryos, Møllgård *et al.* (2017) found that  $\alpha$ -fetoprotein was confined to the lumen of the first blood vessels entering the CNS tissue and the specific marker for cerebral endothelial cell tight junctions, claudin 5, was already present. These observations suggest the presence of a functionally effective blood–brain barrier to protein in the human embryo in the earliest vessels growing into the brain parenchyma.

**Is increased permeability (leakiness) to dyes and small molecular mass markers due to ‘breakdown’ of tight junctions?** Examples can be found in Krizbai *et al.* (2005), Doherty *et al.* (2016) and Reinhold & Rittner





**Figure 11. Tubulocisternal endoplasmic reticulum (TER) in fetal sheep choroid plexus epithelial cells**

A and B, electron micrographs of E60 fetal sheep choroid plexus. Alcian blue in Krebs solution was injected i.v. 10 min prior to fixation. Alcian blue is electron dense and binds to plasma albumin. Particulate precipitate (P in A) is visible within tubular endoplasmic reticulum (TER; dark arrows in B), which extends close to the lateral cell membrane (LM). JC, junctional complex separating the lateral intercellular space from lateral ventricular CSF. Curved arrows in A indicate precipitated Alcian blue on apical cell membrane; open arrows indicate close contact of TER with apical cell membrane, exposed to CSF. From Møllgård & Saunders (1975). C, high voltage double impregnation thick section EM of E60 fetal sheep choroid plexus. Note the extensive network of TER with contacts to CSF surface (uppermost in micrograph) and close association of TER with Golgi complex (G) and mitochondria (M). From Møllgård & Saunders (1977). D, electron micrograph of ultracryosection from E60 fetal sheep choroid plexus immunolabelled for human (HA) 6 nm gold particles and sheep albumin (SA) 12 nm gold particles (arrows). Gold particles labelling each of the albumins are colocalized within the same TER-cistern (star) and a multivesicular body (MVB; inset). Scale bar, 0.2  $\mu$ m. From Balslev *et al.* (1997a). E and F, fetal sheep choroid plexus (E60) double impregnation technique. Profiles of rough endoplasmic reticulum (RER) and TER system. Note TER termination on apical plasma membrane via a caveola (arrowhead in F). MVB, multivesicular body; M, mitochondrion. From Møllgård & Saunders (1977).

(2017). To demonstrate this requires electron microscopy, which is rarely done. In the absence of EM evidence, the 'leakiness' could as well be transcellular via caveoli/vesicular transport rather than being due to disrupted tight junctions. This has been demonstrated when electron microscopy was employed (Povlishock *et al.* 1978; Ben-Zvi *et al.* 2014; Krueger *et al.* 2015; Nahirney *et al.* 2016; Andreaone *et al.* 2017). However, it seems that whether or not changes in the ultrastructure of tight

junctions occur in pathological conditions may depend on the nature of the disorder (Castejón, 2012). An additional potential source of confusion is that it often seems to be assumed that a change in tight junction protein or gene expression is synonymous with tight junction dysfunction (Sun *et al.* 2013; Leclercq *et al.* 2017). Of relevance to blood–brain barrier development is that it seems that transcellular transport may be important in developing cerebral vessels but appears to be suppressed by the

gene major facilitator super family domain containing 2a (Mfsd2a) (Ben-Zvi *et al.* 2014). See also 'Permeability of the paracellular pathway', below.

**Does greater accumulation of a molecule in developing brain and CSF reflect greater permeability of barriers in the developing brain?** Such apparent permeability may be due to the use of brain/plasma or CSF/plasma ratios without allowing for the progressive decline in blood level of a marker following injection: compare Ferguson & Woodbury (1969) in which this was not done and Dziegielewska *et al.* (1979) in which it was. These studies gave very different values for radiolabelled inulin. It might have been hoped that articles such as Ferguson & Woodbury (1969) would have disappeared into the obscurity of unsatisfactory papers, but it was cited recently by Amaraneni *et al.* (2017) implying that it was evidence of a progressive decrease in blood–brain barrier permeability with age. The accumulation of inulin in brain and CSF in the fetal sheep experiments of Dziegielewska *et al.* (1979) did indeed decline with age (although the actual levels were much less than in Ferguson & Woodbury (1969)), but this decline in what we might call 'apparent permeability' was more likely to have been due to the marked increase in CSF secretion (sink effect) that occurs as brain development progresses (see Johansson *et al.* 2008).

**What is the role of astrocytes in formation (initiation) and maintenance of blood–brain barrier properties?** The controversy surrounding the possible role in the formation of tight junctions stems from the paper of Janzer & Raff (1987) and numerous *in vitro* studies of cerebral endothelial cells, which have shown that the presence of astrocytes in the culture system was essential for development of some features of monolayers of cerebral endothelial cells that it was hoped would mimic essential features of the blood–brain barrier. The relevance of *in vitro* studies of these endothelial cells for understanding blood–brain barrier mechanisms *in vivo* seems doubtful, given that cells change their properties and gene expression when isolated (Szmydynger-Chodobska *et al.* 2007; Lyck *et al.* 2009; Zhang *et al.* 2016); this will not be discussed further. Janzer & Raff (1987) claimed that following injection of astrocytes into the anterior eye chamber of the rat or on the chorioallantoic membrane of the chicken embryo, blood vessels formed that were impermeable to injected dye because of the formation of tight junctions between the cells of what were assumed to be blood vessels. However, demonstration of tight junctions requires electron microscopy, which was not performed. When this was done in a replication of the study (Holash *et al.* 1993), it was shown that the dye-containing structures were not blood vessels and no tight junctions were observed. Unfortunately the

Janzer & Raff (1987) paper continues to be cited as evidence of the involvement of astrocytes in the formation of tight junctions in vessels of the developing brain (Abbott, 2002; Engelhardt, 2003; Siegenthaler *et al.* 2013; Rosas-Hernandez *et al.* 2018). This is perhaps an example of when author eminence and the supposed standing of a journal trumps high quality evidence (Vazire, 2017). It seems to be frequently overlooked that no astrocytes have differentiated at the time when the brain is first vascularized and the capillaries are impermeable to even small lipid-insoluble molecules because of the presence of functionally effective tight junctions (Ek *et al.* 2006). Furthermore, capillaries in the subarachnoid space have tight junctions (Balslev *et al.* 1997b), but there are no astrocytes in this location. Also controversial is the role that astrocytes may play in postnatal development and more particularly maintenance of a range of blood brain barrier properties (Abbott, 2002; Obermeier *et al.* 2013; Engelhardt & Liebner, 2014; Saunders *et al.* 2016a).

**Permeability of the paracellular pathway.** The concept of the paracellular pathway arose from experiments of Frömter & Diamond (1972) in which they measured trans-epithelial resistance in a variety of epithelia *in vitro* by passing a microelectrode over the external surface of the epithelium. There was a marked drop in resistance across the epithelium when the electrode tip was over the region between adjacent cells. It was proposed that this indicated a low resistance pathway across the epithelium for water and ion flow. Diamond (1974) later suggested that this was also the route by which small lipid insoluble molecules, such as sucrose, crossed epithelia. The concept was later extended to cerebral endothelial cells. The physical basis for the paracellular pathway is the tight junction component of the junctional complex between adjacent cells of an epithelial interface (Farquar & Palade, 1963). Brightman & Reese, (1969) using horseradish peroxidase and transmission EM showed that horseradish peroxides (actually its reaction product) did not permeate through the tight junctions of either choroid plexus epithelial or cerebral endothelial cells. More recent studies using small lipid insoluble molecules that are visualizable at the EM level (286 Da biotin ethylenediamine and 10 kDa biotin-labelled dextran) showed that these molecules did not permeate tight junctions of cerebral endothelial or choroid plexus epithelial cells in *Monodelphis domestica*, even in the newborn period when the brain is first vascularized and the choroid plexuses are first apparent; rather these molecules traversed plexus epithelial cells by a transcellular route (Ek *et al.* 2003, 2006; Liddelow *et al.* 2009). Thus in respect of small lipid-insoluble molecules, Diamond's (1974) proposal appears to be incorrect although it is still widely believed as a fundamental property of epithelia and cerebral endothelium (Abbott



et al. 2010). In the case of water and ions there is no direct evidence that they cross epithelial interfaces through a paracellular pathway as proposed by Frömter & Diamond (1972) as water and ions cannot yet be visualized at a sufficiently high resolution to demonstrate this. An important limitation of the experiments of Frömter & Diamond (1972) is that the dimension of the tip of the microelectrodes used was about  $5\ \mu\text{M}$ . This compares to the intercellular space at the level of tight junctions, which is measured in nanometres, if not zero where adjacent cell membranes are fused. Thus an alternative explanation for the low resistance pathway is that there is an intracellular pathway close to the border of the cell membrane between adjacent cells. Diamond (1974) seems to have acknowledged this possibility as he suggested that in some epithelia the low resistance pathway might be due to 'leaky' cells, although he thought this would be exceptional. The studies of Ek et al. (2003, 2006) require independent replication, preferably in several different species, but they indicate that the earliest vessels growing into the brain are structurally well enough developed for their tight junctions to be impermeable to even very small molecules. Similarly, in the early developing choroid plexus the paracellular route appears to be closed to small lipid-insoluble molecules. Thus a low resistance trans-cellular pathway across epithelial cells for ions and water, such as in the choroid plexus, is a plausible alternative explanation to the paracellular route envisaged by Frömter & Diamond (1972); these alternatives will only be resolved when suitable high resolution microscopical methods become available. The current dependence on *in vitro* systems (the permeability properties of which are generally not representative of those in intact vessels, Curry, 2005), modelling (Curry, 2005), cartoon biology (Gunzel, 2017) and deductions from pathological conditions (Mankertz & Schulzke, 2007) to support the functional importance of the paracellular pathway may be misplaced.

### Different animal species used for brain barrier studies

*For a large number of problems, there will be some animal of choice on which it can be most conveniently studied* (Krogh, 1929)

Effective research not only depends upon the convenience of the species but also on its relevance to the problem being studied. Thus the animal species to be chosen depends to a large degree on the questions asked. In developmental studies it is often a trade-off between the limits imposed by the size of the species (for example where adequate physiological monitoring is essential) and the requirement to study as early stages of development as possible.

Table 4 provides information about developmental milestones and ages at which experiments have been

**Table 4. Developmental milestones of mammalian species commonly used for blood-brain and blood-CSF barrier studies**

	Group I		Group II		Group III		Group IV		
	Short gestation, at birth cortex undifferentiated		Short gestation, at birth neurogenesis near complete		Intermediate gestation, brain more mature than Group II		Long gestation, larger brain at birth neurogenesis & gliogenesis mostly complete		
	Opossum	Wallaby	Mouse	Rat	Rabbit	Guinea	Sheep	Pig	Human
Days	14	28	20–21	21–22	30–32	67–70	147	115	266
post-conception									
Neural tube closure	E10.5*	~E18 <sup>†</sup>	E9–E9.5 <sup>‡</sup>	E11 <sup>‡</sup>	E9 <sup>‡</sup>	14 <sup>‡</sup>	E21 <sup>‡</sup>	E17–E18 <sup>‡</sup>	E26
Published permeability <sup>§</sup> studies	P0 <sup>¶</sup> P5	P0–3	E11 <sup>¶</sup> E12	E13	E28–30 <sup>a</sup>	E60–65 <sup>b</sup>	E21 <sup>¶</sup> E50	E20 <sup>¶</sup>	E35 <sup>¶</sup>

\*McCready (1938). <sup>†</sup>Ullmann et al. (1997). <sup>‡</sup>Evans & Sack (1973). <sup>§</sup>Permeability studies include blood-brain and blood-CSF barriers. <sup>¶</sup>Blood-brain barrier impermeability to plasma proteins demonstrated by immunohistochemistry only. Data sources for permeability studies: opossum (*Monodelphis domestica*), Knott et al. (1997); tammar wallaby (*Macropus eugenii*), Dziegielewska et al. (1986); mouse, Bauer et al. (1995); rat, Johansson et al. (2006); sheep, Evans et al. (1974); Dziegielewska et al. (1979, 1980b); Reynolds et al. (1983); pig, Cavanagh & Møllgård (1985); human, Møllgård et al. (2017); rabbit, <sup>a</sup>Sandberg et al. (1996); guinea pig, <sup>b</sup>Bissonette et al. (1991).

performed in the key mammalian species that have been used for developmental brain barrier studies. We have divided the species into four groups based on stages of brain development at birth. Group I are marsupial species, which are born with a two-layered undifferentiated neocortex; their choroid plexuses are only just beginning to appear and all four are present within a week post-natal. These animals are very small (10 mm crown–rump length) at birth but have the considerable advantage compared to eutherian mammals that they are born at such an early stage of development that they can be studied without having to work *in utero*. They also survive much better than rodents at equivalent stages of brain development. Tammars are seasonal breeders and usually have only one young; they require substantial infrastructure for maintaining a colony. *Mono-delphis* will breed all the year round and have multiple young that are exposed on the mother's abdomen; they are pouchless marsupials. They can easily be bred within a standard animal house, although they require a higher room temperature and humidity than rodents (Fadem *et al.* 1982). Some have questioned the value of marsupial species for developmental studies on the grounds of lack of research tools compared to rodents. However the genomes of both species have now been sequenced (Mikkelsen *et al.* 2007; Renfree *et al.* 2011) and in our experience antibodies to human antigens cross-react well with proteins in these species.

Group II are neonatal rats and mice, which are as suitable as marsupials for barrier studies at later stages of development. At birth the neurogenesis is mostly complete while gliogenesis is beginning. *In utero* studies are more difficult technically because of small size, fragility and difficulty of maintaining fetuses in a physiologically normal state. Group III animals are appreciably larger at birth and more advanced in brain development. *In utero* experiments are also difficult although a few studies have been published (e.g. Bissonnette *et al.* 1991; Sandberg *et al.* 1996). Group IV is the sheep fetus, which since it was introduced by Barcroft and Barron (Barcroft *et al.* 1936, 1940) has been the traditional species for a whole range of developmental physiological studies. Sheep placenta is made up of around 100 cotyledons, each with its own circulation (Dawes, 1968). Thus access to the fetal circulation can be obtained with minimal disruption of the placental circulation, which is a significant problem in rodents, where there is a single placenta for each embryo. Permeability studies in fetal sheep under monitored and reasonably well-controlled physiological conditions have been possible as early as E50 (Evans *et al.* 1974). Most blood–brain barrier studies have been conducted at older ages (E60: Evans *et al.* 1974; Dziegielewska *et al.* 1979, 1980*bb*, 1991) or later (e.g. E87 to term: Stonestreet *et al.* 1996). The monitored studies in fetal sheep are relatively expensive and require more space and instrumentation,

but they provide important background validation to studies in species where monitoring has so far not been technically possible.

Not included in Table 4 are non-mammalian species such as chick and zebrafish embryos. These have the considerable advantages of accessibility at very early stages of brain development. Zebrafish have the so-far-unique advantage of being transparent so various processes can be visualized real-time with time-lapse microscopy (Chang & Sive, 2012; Umans *et al.* 2017). More detailed information on brain development milestones in some of these species is to be found in Clancy *et al.* (2001, 2007), Clowry *et al.* (2010) and Semple *et al.* (2013).

In conclusion, because each species comes with inherent limitations, it is preferable to study barrier problems in several animal species. This extends the range of developmental ages that can be studied and provides complimentary evidence on a particular question, rather than mere replication. This is an approach that has been described as 'triangulation' (Munafò & Smith, 2018).

### Future studies

Further work will be required to resolve the controversies outlined above. In addition, there are several topics that have been barely touched on in studies so far. These include when the proposed cerebral lymphatic system (Johnston *et al.* 2004; Iliff & Nedergaard, 2013) develops (Koh *et al.* 2006) and whether the 'glymphatic' system (Plog & Nedergaard, 2017) is functional in the developing brain. Major areas for future research are the following.

**Drugs that enter the developing brain: do they affect brain development and behaviour?** The efflux mechanisms summarized above limit the entry of drugs into the adult brain. But the effectiveness of these protective mechanisms in the developing brain when drugs are administered to pregnant women or to neonates is largely unknown. Systematic studies in the human fetus that define which efflux transporters are present in the developing brain and when they appear (e.g. Møllgård *et al.* 2017) are required as a prerequisite for functional studies of these transporters in an appropriate animal model. Fortunately it seems likely that only a limited number of the ABC transporters are involved. Thousands of drugs and toxins are kept out or eliminated from the brain; this is because many drugs are substrates for individual transporters. Thus if key drugs that are known to be substrates for each efflux transporter are selected, then the effectiveness of each transporter can be assessed at different stages of brain development. However, this assessment is complicated by a number of factors. Any one drug may be a substrate for more than one efflux

transporter. Many of the transporters are also functional in the placenta. Thus placental function needs to be taken into account when assessing the possible entry of drugs into the brain. On the other hand for prematurely born infants that have lost the protection of the placenta early, it is possible that the brain will be more susceptible to the entry of drugs administered in the neonatal period. There is some evidence, although as yet controversial, that some transporters up-regulate in the face of continued exposure to their substrates. Thus for pregnant women on regular medication, for example anti-epileptics, this may affect the entry of drugs into the fetal brain. Once it is established which classes of drugs can enter the developing brain and when, a large field will open up for systematic studies of the short- and long-term consequences of drug exposure in pregnancy and the neonatal period. At present only limited studies have been carried out and these are often poorly designed, resulting in unnecessary alarm in patients. To give a few recent examples: poor quality epidemiological studies of paracetamol in pregnancy (Stergiakouli *et al.* 2016, see comments in Beale, 2017; Saunders & Habgood, 2017); clinically improbable doses of paracetamol in pregnant mice (Hay-Schmidt *et al.* 2017) and administration of penicillin to mice during a large proportion of pregnancy and the postnatal period (Leclercq *et al.* 2017) in which there was a lack of randomization, lack of blinding of behavioural studies, dubious statistical analysis and dubious interpretation of results of tight junction protein expression.

**Composition, origin and functions of CSF in mammalian embryos.** It is worth emphasizing the likely importance of CSF in the development of the CNS. Consideration of this can conveniently be divided into two stages. (a) after the closure of the neural tube, which traps amniotic fluid within the tube and before choroid plexuses appear. Its composition is subsequently modified although different emphasis has been placed on the importance of materials emanating from the neuroepithelium (Zappaterra *et al.* 2007; Chau *et al.* 2015) and those entering from outside the CNS (Bueno *et al.* 2014; Gato *et al.* 2014). (b) the period from differentiation of the choroid plexuses onwards. There is evidence that proteins are transferred from the circulating blood to the CSF across the plexus epithelial cells (see 'Transfer of plasma proteins across choroid plexus epithelial cells', above) but other constituents of the CSF are likely to originate from the choroid plexuses themselves or from the neuroepithelial tissues lining the ventricular system. It is worth emphasizing that in the first stage (after the closure of the neural tube) the neural tissue is not vascularized and in the early period of the second stage (from differentiation of the choroid plexuses onwards) there is little vascularization of the

CNS; it is only after birth, for example in rodents, that the neocortex develops most of its vasculature (Caley & Maxwell, 1970). Thus non-vascular sources of the constituents of CSF dominate in the early stages of brain development. Features of brain development that have so far been studied in the context of specific aspects of brain development are neurogenesis (Alonso *et al.* 2011; Lehtinen & Walsh, 2011; Johansson, 2014) and cell proliferation (Miyan *et al.* 2006; Lehtinen *et al.* 2011). Zappaterra & Lehtinen (2012) have reviewed a range of developmental processes in which CSF constituents may be involved.

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## Additional information

### Competing interests

None declared.

### Author contributions

All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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